Novel Exotoxic Principle(s) Produced by the Toxic Dinoflagellate *Alexandrium minutum:* Effects on Brine Shrimp (*Artemia salina*)and Larval Fish (*Rhombosolea taparina*)

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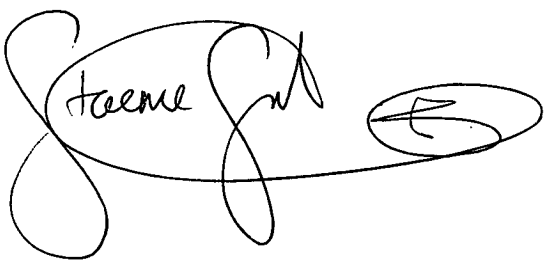

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Abstract

A number of species of the dinoflagellate genus *Alexandrium* have been implicated in the production of Paralytic Shellfish Poisoning (PSP) toxins but occasionally have also been associated with the mortality of both cage-reared and wild finfish in such places as the Faroe Islands (*A. tamarense*) (Mortensen 1985), Taiwan (*A. minutum*) (Su *et al.* 1993) and Egypt (*A. minutum*) (Halim 1996).

In the present work exocellular toxicity was investigated in two species of Australian dinoflagellates, *A. minutum* and *Gymnodinium catenatum*. Both are known producers of neurotoxic PSP toxins, but these toxins are only known to exist endocellularly. Investigations took the form of animal based biological assays using the brine shrimp, *Artemia salina*, and juvenile greenback flounder, *Rhombosolea taparina*, which were exposed to whole cell and cell-free cultures of the algae. Bioassays determined the time to mortality for both species, while pathological changes in major tissues and changes in physiochemical characteristics of the blood were characterised only in the flounder.

Even though *Artemia* did not feed on toxic dinoflagellate cells of *G. catenatum* and *A. minutum*, cultures of these two species were found to kill *Artemia* within 24 hours. However, only *A. minutum* culture-filtrate was toxic to *Artemia* whereas *G. catenatum* culture-filtrate was not. Likewise, culture-filtrate of unialgal *G. catenatum* cultures was found to be non-toxic to flounder with no pathological changes in gills or other tissues observed. This disproves the suspected involvement of *G. catenatum* as the cause of clubbing necrosis gill syndrome (Clark *et al.* 1997) documented in farmed Atlantic salmon in Tasmania. In contrast, the culture-filtrate of unialgal *A. minutum* cultures was found to be highly toxic to flounder. *Artemia* and flounder mortality occurred within 24h of exposure to full strength *A. minutum* culture-filtrate. Flounder exhibited mild to severe histopathological gill changes including: swelling, degeneration and sloughing of the respiratory epithelium. The primary lamellar epithelial cells were so swollen in some areas that they displaced the respiratory epithelial cells into the interlaminar region. Also apparent were varying degrees of swelling, vacuolation, degeneration and cytoplasmic shrinkage of the gill chloride cells and hypertrophic or discharged mucocytes. Also common were antemortem blood clots in the heart ventricular tissue. Changes in blood physiochemical characteristics of flounder were also found with increases in blood

potassium inducing a state of hyperkalemia. Hyperkalemia affects the contractile functioning of the heart, causing bradycardia and atrial standstill and can lead to cardiac arrest. Increases in gill-associated succinic dehydrogenase activity were observed but only when flounder were exposed to 60% *A. minutum* culture-filtrate mixed with 40% sterile seawater. No similar effect was observed in *Artemia* or flounder from exposure to seawater containing purified gonyautoxins (GTX) 1-4 in concentrations equivalent to the normal endocellular levels. Activity from the exocellular medium of *A. minutum* similar to that exhibited by Neurotoxic Shellfish Poisoning (NSP) toxins was revealed by neuroblastoma tissue culture assays (Manger *et al.* 1993), but neuroreceptor binding assays (Van Dolah *et al.* 1994) on the same material failed to detect brevetoxins. Lipid soluble *A. minutum* extracts also failed to show conclusive evidence for cytotoxicity to erythrocytes or gill tissues or toxicity to *Artemia*. This would discount the involvement of the fast acting spirolides (reported from *A. ostenfeldii*), which are lipophilic (Cembella *et al.* 1999).

Endo/exotoxicity of *A. minutum* was followed over the growth cycle of the dinoflagellate in batch culture. Total Sodium Channel Blocking (SCB) toxin concentrations (STX equivalents) and profiles of individual GTX 1-4 components (endocellular only), as well as toxicity of the culture-filtrate to *Artemia* were examined. SCB activity was monitored by neuroreceptor binding assay. Peaks in toxicity of the exocellular medium appeared concurrent with peaks in the endocellular toxicity of GTXs (both occurring in early to late lag phase and declining as time progressed). The SCB toxicity of the medium was found to be three orders of magnitude higher (3.7-10.2 pg STX equivalents $cell^{-1}$) than the endocellular SCB toxicity (169-610 fg STX equiv. $cell^{-1}$). This is one of the first reports conclusively demonstrating SCB toxicity in the exocellular medium of a dinoflagellate

Endocellularly, the gonyautoxins GTX_2 and GTX_3 were dominant early in culture growth (36-64 mole%, epimeric total on day 48) but as time progressed GTX_1 and GTX_4 became increasingly important (58-93 mole %, epimeric total on day 182). Antibiotic treatment of dinoflagellate cultures reduced bacterial levels by up to 93% but this did not appear to affect SCB toxicity, although it did decrease toxicity of the exocellular medium to *Artemia*, which was highest later in culture growth. Toxicity of the *A. minutum* culture-filtrate towards *Artemia* did not appear to be correlated with exocellular or endocellular total SCB toxicity nor was it correlated with any individual toxic GTX fraction. It is concluded that, in addition to a

Sodium Channel Blocking agent, *A. minutum* is producing a cytotoxic ichthyotoxin that is not a PSP toxin. This exotoxic principle is heat labile (reduced toxicity to *Artemia* above 80°C), but is not a gonyautoxin or brevetoxin.

In fish *A. minutum* culture medium produces severe pathological gill lesions which resemble some of those caused by the raphidophyte *Chattonella marina* (Endo *et al.* 1985) which is also associated with the NSP syndrome. Although damage to fish gills upon exposure to *C. marina* is thought to be due to the production of highly reactive oxygen radicals (Tanaka *et al.* 1992) this was ruled out as the cause of histopathology in this study as oxygen radicals are short lived and appear to only occur in photosynthetically active cultures. The toxic principle(s) of *A. minutum* remained active in the culture-filtrate even after the cells were removed and stored frozen, in the dark for several weeks.

The present work on novel ichthyotoxins in *A. minutum* culture medium adds to preliminary reports of such activity by both this species (Bagoien *et al.* 1996) as well as *A. tamarense* (Hansen 1989, Ogata and Kodama 1986), suggesting that this phenomenon may be more widespread.

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Chapter 1: General Introduction

1.1 Microalgal Blooms and Impacts: A General Overview

Marine microalgae are over three billion years old and it is probable that blooms of them have existed for as long (Smayda, 1990). The exact cause of blooms is still poorly understood however they normally comprise a single microalgal species which outcompetes other species and becomes locally dominant in the water column in terms of numbers and biomass. Bloom formation and subsequent collapse tends to produce a process of successional rise and decline that appears to be essential in terms of the energy and trophic dynamics of the marine food web particularly as microalgae are the primary link between the energy of solar radiation to a form useable by other non-photosynthetic organisms (Richardson, 1989). However, at times, microalgal blooms can have serious negative effects on the local marine environment causing major losses in aquaculture and wild fisheries as well as human health impacts.

Among the 4375 species of extant marine microalage (Sournia *et al.*, 1991), some 300 species (Hallegraeff *et al.*, 1995) can occur in such high densities that they actually discolour the surface of the water. These harmful algal blooms (HABs) can also induce states of stress in other marine organisms by producing anoxic conditions from high respiratory rates at night or during periods of low light, bacterial decomposition of the microalgal cells when the bloom is in decline, physical clogging of the gills of fish, or mechanical damage of exposed tissues by particularly spinose species such as the diatom *Chaetoceros convolutus*. Also, approximately 40 species (with the number increasing with increasing research) of marine microalgae have the ability to produce potent toxins that can kill marine organisms or find their way through intermediate vectors of the food chain all the way to humans (Hallegraeff *et al.*, 1995). In fact, toxigenic algae may exert disproportionately large impacts on the members of the local marine or estuarine community as algal toxins can flow through aquatic food chains in a manner analogous to carbon or energy (Smayda, 1992). Toxin producing microalgae, particularly those of the Dinophyceae, will be the focus of this thesis.

The first written reference to a harmful algal bloom is thought to be in the Bible (1000 years BC) in Exodus (7: 20-21) "*all the waters that were in the river were turned to blood. And the fish that were in the river died; and the river stank, and the Egyptians could not drink of the*

water of the river".....

Fossil evidence also indicates a mass mussel death due to an algal bloom 140 million years ago in the now Baltic sea region (Surlyk & Noe-Nygaard 1988 in: Richardson, 1989). History indicates that North American indians refrained from eating shellfish when algal blooms had occurred, evidenced by bioluminesence in the water (Richardson, 1989, Hallegraeff *et al.*, 1995). While in more recent history, toxic microalgal blooms have caused massive economic losses due to the increase in the last two decades of aquaculture. For example the hemolytic (and neurotoxic?) microalga *Chattonella antiqua* was responsible for the loss of $US500 million worth of yellowtail fish in the Seto inland sea, Japan (Okaichi, 1989), while $NZ12 million (>800 tonnes) (MacKenzie, 1991) worth of chinook salmon was killed in Big Glory Bay, New Zealand, by another hemolytic microalga, *Heterosigma akashiwo*. (Chang *et al.*, 1990), stock losses of 27 tons of rainbow trout and salmon killed by *Alexandrium tamarense* (neurotoxic) in the Faroe Islands (Mortensen, 1985), and 700 tonnes of Atlantic salmon killed by the hemolytic species *Chrysochromulina leadbeateri*, in Northern Norway (Tangen, 1991). Here in Australia in 1996 a massive kill of tuna resulted from a bloom of *Chattonella* sp. Tuna livers were found to contain high concentrations of a Brevetoxin-like (hypopolarizing neurotoxin) substance, causing rapid death and an estimated loss of $AUS 45 million.

1.2 Phycotoxins

Toxins produced by marine microalgae (phycotoxins) are among the most potent non-proteinaceous poisons known (Tosteson *et al.*, 1989). Twenty two dinoflagellate species are known to produce toxins (Steidinger & Baden, 1984; Tosteson *et al.*, 1986) including both water and lipid soluble moieties, which have neurotoxic, hemolytic, hepatotoxic and gastrointestinal inflammatory activities. Only relatively few phycotoxins have been characterized chemically with the most widely documented toxins causing five known toxic syndromes: Paralytic (PSP), Diarrhetic (DSP), Amnesic (ASP), Neurotoxic (NSP) and Ciguatera. The phycotoxins involved in these syndromes are mostly produced by dinoflagellates (Ciguatera is caused by a benthic dinoflagellate) except ASP which is caused by toxic diatoms. Cyanobacteria also produce several toxic syndromes mostly confined to freshwater systems (see Table 1.1). Chemically, the most common marine phycotoxins constitute forms of alkaloids, polyethers, substituted amines (Plumley, 1997), superoxide and/or hydroxyl radicals (Oda *et al.*, 1992, Tanaka *et al.*, 1992), lipoteichoic acids with hemagglutinin activity (Ahmed *et al.*, 1995), pentacyclic derivatives with fused azines (Seki

Table 1.1: Six of the most common phycotoxic syndromes, causative toxins, algal species responsible and symptoms of intoxication.

Toxic Syndrome	Type Toxin	Species Responsible	Symptoms of intoxication
Paralytic Shellfish Poisoning (PSP)	saxitoxin gonyautoxins neosaxitoxin, decarbamoylsaxitoxin	*Alexandrium catenella* *A. minutum* *A. tamarense* *Gymnodinium catenatum,* *Pyrodinium bahamense-* *var compressum*	tingling sensation or numbness around lips, gradually spreading from face to neck, prickly sensation in fingers and toes, headache, dizziness, nausea, vomiting, diarrhoea, muscular paralysis, pronounced respiratory difficulty, choking, death via respiratory paralysis may occur within 2-24 hrs post toxin ingestion.
Diarrhetic Shellfish Poisoning (DSP)	okadaic acid DTX1, DTX2	*Dinophysis sp.* *Prorocentrum lima*	diarrhoea, nausea, vomiting, abdominal pain, chronic exposure may promote tumor growth in gut.
Amnesic Shellfish Poisoning (ASP)	domoic acid	*Pseudo-nitzschia multiseries,* *P. australis,* *P. pseudodelicatissima*	after 3-5 hrs: nausea, vomiting, diarrhoea, abdominal cramps, decreased reaction to deep pain, hallucinations. confusion, short-term memory loss, seizures.
Ciguatera	gambiertoxin CTX1 CTX2 CTX3	*Gambierdiscus toxicus,*	diarrhoea, abdominal pain, nausea, vomiting, numbness and tingling of hands and feet, reversal of thermal touch sensation, loss of balance, low blood pressure and heart rate, rashes, death via respiratory failure, neurological symptoms may last months or years.
Neurotoxic Shellfish Poisoning (NSP)	brevetoxin	*Gymnodinium breve*	after 3-6 hrs: chills, headache, diarrhoea, muscle weakness muscle and joint pain, nausea, vomiting, paraesthesia reversed thermal touch sensation, respiratory difficulty, visual difficulty, speech and swallowing difficulty.
Cyanobacterial Toxin Poisoning	anatoxins cylindrospermopsin saxitoxin, neosaxitoxin microcystins, nodularin	*Anabaena circinalis,* *Microcystis aeruginosa,* *Nodularia spumigena*	hepatotoxins: hepatic enlargement, necrosis, hemorrhage hemorragic enteritis, pulmonary edema. neurotoxins: as for PSP's dermatotoxins: skin rashes and lesions

et al., 1995) and fatty acids and galactolipids with hemolytic activity (Yasumoto *et al.*, 1987, Yasumoto *et al.*, 1990). Many more marine phycotoxins have been documented, with listings of their effects upon marine fauna, however ellucidation of their specific chemical nature is absent (Ogata & Kodama, 1986, Gentien & Arzul, 1990, Smolowitz & Shumway, 1997).

Phycotoxins are viewed by many researchers as secondary metabolites. Vining (1990) classes secondary metabolites as compounds that do not fulfil a role in intermediary metabolism and tend to encompass a chemically diverse array of substances (Plumley, 1997). The specific role of secondary metabolites is unclear but encompasses UV light protection, nutrient storage, antiherbivory (i.e. toxic) allelopathy, symbiosis promotion and metal scavenging. Saxitoxins for example, have been proposed as Nitrogen storage molecules by Anderson *et al.* (1990) (saxitoxin is 33% N on a molecular weight basis), an antipredation compound by Haney *et al.* (1995) due to their extreme toxic potency, roles in DNA metabolism by Mickelson & Yentsch (1979) and as a shunt to reduce NH_4 toxicity in eutophic environments (Cembella, 1998). Secondary metabolites are also produced in very small quantities under optimal conditions but production is greatly enhanced when conditions limit growth. This situation is the same as that observed for many of the HAB toxins particularly PSP toxins. However, if the sheer molecular complexity, common to all HAB toxins, is taken into account, it is highly unlikely that they have no function as they are likely to be the end product of very elaborate biosynthetic pathways involving some unique and specialised reactions. At present little is known of the specific biochemistry of toxin synthesis of many HAB toxins.

Many species of cyanobacteria are responsible for producing toxins but these are normally confined to freshwater systems and thus not treated in detail here. Toxic strains within the species *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Microcystis*, *Nodularia*, *Nostoc* and *Oscillatoria* are responsible for the majority of toxic episodes although there are over 30 species of cyanophytes that can be associated with toxic blooms (Skulberg *et al.*, 1993). Neurotoxic alkaloids (anatoxins) are produced by species of *Anabaena*, *Aphanizomenon*, and *Oscillatoria* while Paralytic Shellfish Poisoning (PSP) toxins normally attributable to marine dinoflagellates can be produced by *Anabaena*, *Aphanizomenon* and *Lyngbya*. Hepatotoxic (toxic to liver cells) peptides such as microcystin and nodularin have been observed being produced by *Anabaena*, *Microcystis*, *Nodularia*, *Nostoc* and *Oscillatoria* while species of *Cylindrospermopsis* and *Umezakia* can produce the hepatotoxic alkaloid, cylindrospermopsin (Carmichael in Hallegraeff *et al.*, 1995). In general however,

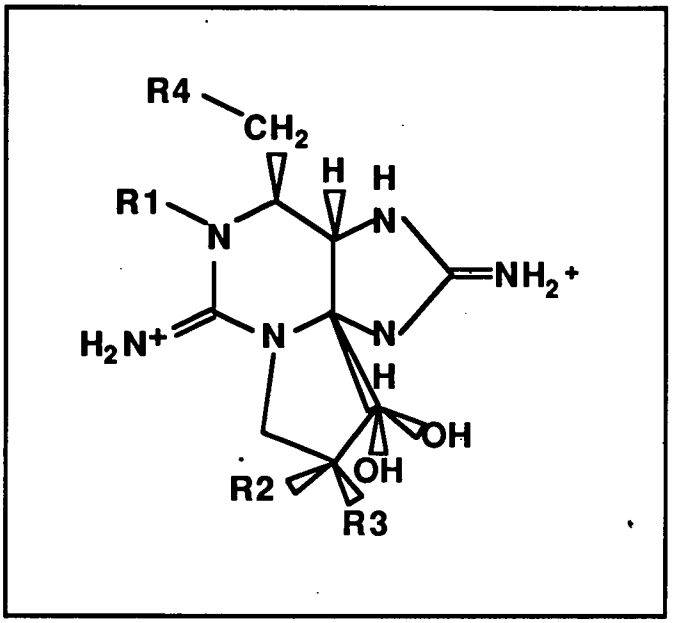
the structures of freshwater cyanophytic toxins are much more diverse than those produced by dinoflagellates in marine systems and include alkaloids, phosphate esters, macrolides, chlorinated diaryllactones and penta/heptapeptides (Plumley, 1997). As with the majority of toxins produced by marine algae all these have either neurotoxic, hepatotoxic or dermatoxic activities (Carmichael & Falconer, 1993).

1.3 Paralytic Shellfish Poisons: The Saxitoxins

The group of phycotoxins known collectively as the PSP toxins are a group of low molecular weight (saxitoxin = 299 MW), tetrahydropurine compounds (Schantz, 1986, Cembella, 1998) typified by the parent molecule saxitoxin (STX) (Fig. 1.1), with approximately two dozen naturally occurring derivatives that have been found among various organisms (Shimizu, 1996). STX contains two guanidinium groups with the STX analogues differing in N-1-hydroxyl, 11-hydroxysulfate and 21-N-sulfo substitutions, including epimerization at the C-11 position (Schantz, 1986, Hall *et al.*, 1990, Cembella, 1998). Based upon these substitutions, the PSP compounds are now classified as the high potency carbamate toxins (STX, NEO [neosaxitoxin], GTX_{1-4} [gonyautoxins 1, 2, 3, 4]) the low potency N-sulfocarbamoyl group (B_1, B_2, C_{1-4}) and decarbamoyl derivatives ($dcGTX_{1-4}$, dcNEO, dcSTX) (Cembella, 1998) (Fig. 1.1). The PSP toxins are endocellular and not known to be actively excreted into the water column (Oshima, pers. comm.)

The PSP toxins exert their first order toxic effects by binding to certain classes of biological receptors and in most cases, these receptors are exclusively the voltage-gated sodium channels of excitable membranes (Cembella *et al.*, 1995). PSP toxins have practically no other biological actions, and their systemic effects in whole organisms can be explained almost entirely by their cellular effect of blocking the sodium channel (Kao & Hu, 1988). The binding is on a 1:1 stoichiometric basis (Narahashi, 1988), is reversible (Hall & Reichardt, 1984) and causes no change in the resting membrane potential of the cell. The dynamics of binding and subsequent blockage of the channel itself are poorly understood. However, the 7, 8, 9 group on the STX parent molecule has been identified as the biologically active group blocking the channel itself in a positively charged form (Kao *et al.*, 1983, Kao & Hu, 1988). Many researchers indicate that the active guanidinium group is attracted to the fixed negative charges around the external orifice of the sodium channel (which are present in a higher density around the orifice of the sodium channel membrane) (Kao & Nishiyama, 1965, Kao & Walker, 1982,

Narahashi, 1988), particularly site 1 (G. Doucette, pers comm.), with H-bonding between the



			Carbamte toxins	N-sulfo-carbamoyl toxins	Decarbamoyl toxins	Deoxydecarbamoyl toxins
R1	**R2**	**R3**	**R4: $CONH_2$**	**R4: $CONHSO_3^-$**	**R4: OH**	**R4: H**
H	H	H	STX [2045]	GTX5 [350]	dcSTX [1220]	doSTX [-]
OH	H	H	neoSTX [1038]	GTX6 [180]	dcneoSTX [-]	
OH	H	OSO_3^-	GTX1 [1638]	C3 [18]	dcGTX1 [-]	
H	H	OSO_3^-	GTX2 [793]	epiGTX8 [16]	dcGTX2 [530]	doGTX2 [-]
H	OSO_3^-	H	GTX3 [2234]	GTX8 [430]	dcGTX3 [990]	doGTX3 [-]
OH	OSO_3^-	H	GTX4 [673]	C4 [57]	dcGTX4 [-]	

Fig 1.1: Structures and toxicities of paralytic shellfish toxins. Mouse intraperitoneal toxicities in MU/μmole are given in parentheses (after Oshima *et al.*, 1988, Hallegraeff *et al.*, 1995).

hydroxyl groups and deprotonated carboxylate functions in the channel protein adding to the binding process (Kao & Hu, 1988). Hence PSP toxins are only effective in blocking sodium passage from the external membrane surface and are impotent when applied to the internal membrane surface (Narahashi, 1988). Actual blockage of the sodium channel orifice leading to the pharmacological expression of nerve disfunction is hypothesised to simply be a case of physical obstruction (Kao & Nishiyama, 1965).

The blocking of the sodium channel in neuronal membranes causes normal neuron function to

cease and this can occur in the presence of nanomolar concentrations of STX (see Fig. 1.2). Blockage of the sodium channel in neurons does not allow the influx of sodium ions that normally produces the wave of depolarization, or action potential, along the axonal membrane of a neuron. Hence the membrane becomes hyperpolarized at -90mV (equilibrium potential for Potassium as described by the Nernst equation as it is still permeable to Potassium) rather than moving from the resting membrane potential of -80mV to the depolarized potential of +50mV generating the rising phase of the action potential. Brevetoxins (PbTxs) act in in a similar manner to STXs although rather than blocking the channel and effectively closing it, binding of PbTxs occurs at site 5 on the sodium channel orifice and keeps the channel open thus hypopolarizing the membrane at the equilibrium potential for sodium (+50mV).

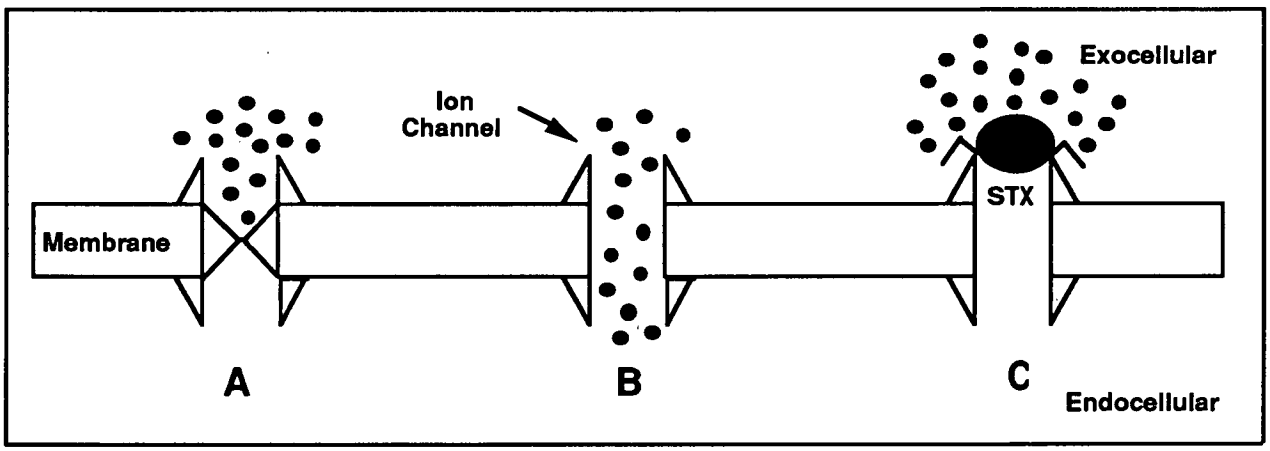


Figure 1.2: Diagramatical representation of sodium channel function and blockage with STX interacting with the membrane of a neuron. A = normal closed ion channel with sodium (•) unable to pass, B = ion channel opened due to voltage step and sodium able to pass through membrane, C = normal open channel after voltage step but channel physically blocked by STX bound to outer surface with sodium unable to pass through membrane.

The microalgae containing STX include three morphologically distinct genera of dinoflagellates. In these organisms the epimeric pairs that constitute the bulk of the STX family of toxins are seldom encountered singly, an extract containing one will generally be found to contain some amount of its epimer, even though this amount may be small. This is for the most part due to the ease with which the 11-hydroxysulfate group spontaneously epimerizes (Schantz, 1986). There is a predominance in equilibrated solutions *in vitro* of 11 α-epimers. However, the ratio of 11 β-epimers to the corresponding 11 α-epimers is consistently higher in fresh dinoflagellate extracts and lower in older extracts, suggesting that the 11 β-epimers are

the principle or only forms synthesised in the organism and that 11 α-epimers arise through epimerization (Schantz, 1986).

The specific biosynthetic pathways for STX synthesis are still largely unclear. There appears to be a fundamental structural similarity between the tetraperhydropurine STX (a guanidine derivative) and common purines of intermediary metabolism suggesting that the PSP toxins may be produced by modifications of the conventional pathway of purine synthesis (Cembella, 1998). However, based on studies using the incorporation of labelled precursors, there appears to be no evidence for a biosynthetic link between purine and STX metabolism. Shimizu *et al.* (Shimizu *et al.*, 1985) determined the basic biosynthetic pathway for production of the perhydropurine ring to form STX over 10 years ago, although some modifications have been added since that time to the basic "bow and arrow" scheme of PSP toxin biosynthesis (Shimizu, 1996). The most current research indicates that the three fundamental units in STX biosynthesis are considered to be a high energy CH_3-group donor (S-adenosyl-methionine: SAM), a two carbon unit (acetate) and an amino acid precursor (Argenine: ARG), although the exact number of enzymatic steps and sequence of the biosynthetic reactions is still uncertain (Plumley, 1997, Cembella, 1998).

1.4 Neurotoxic Shellfish Poisons: The Brevetoxins

The group of toxins termed brevetoxins (PbTxs) are lipid soluble, chemically labile, (Hallegraeff *et al.*, 1995, Wright & Cembella, 1998) *trans*-fused polyether ladder toxins (Baden *et al.*, 1995) (Fig. 1.3) comprised of nine known structural species (Fig. 1.4) (Baden & Trainer, 1993), although there undoubtedly exist other undescribed derivatives (Hallegraeff *et al.*, 1995). The "ladder-frame polyether" toxins also includes the ciguatera toxin complex (ciguatoxins, maitotoxin, gambieric acids) and yessotoxin (Fig. 1.3). All are fused polyethers that do not posses an obvious backbone chain but consist of ether rings linked together in a rigid ladder-frame structure. No remotely similar structures exist in nature and the chemical architecture of these apparently highly conserved molecules is unique to the biosynthetic products of marine dinoflagellates (Wright & Cembella, 1998).

Specifically, PbTxs bind to site 5 (unlike STX's that bind to site 1), on either the internal or external orifices of neuronal, voltage sensitive sodium channels, inducing a channel-mediated sodium ion influx (whereas STX's block sodium ion influx on the external surface only)

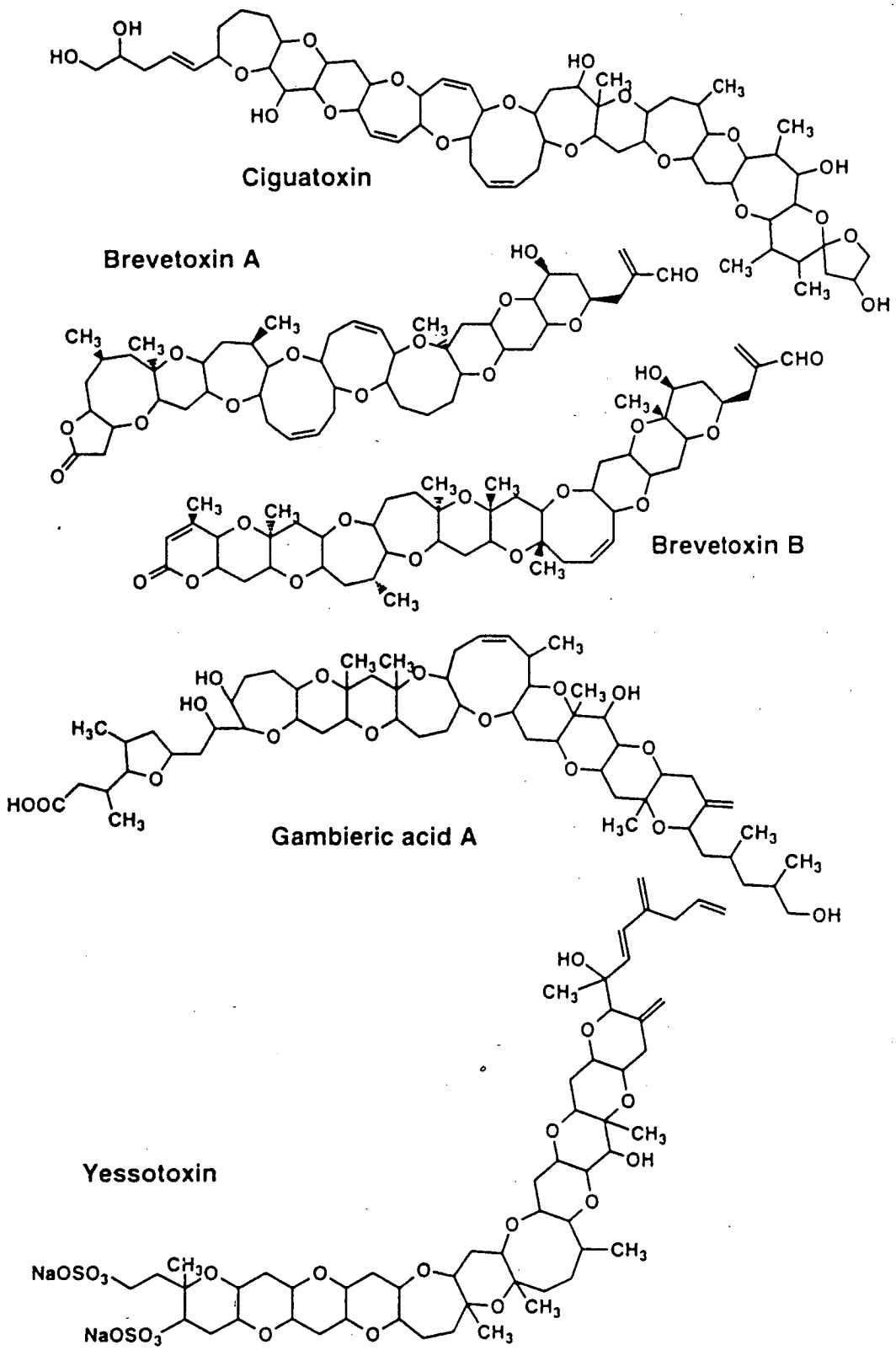


Figure 1.3: Selected examples of ladder-frame polyether toxins (from: Wright & Cembella 1998).

(Baden & Trainer, 1993). The binding, opening and sodium ion influx causes a partial but persistent depolarization of the neuronal membrane of about 30-40mV (Baden & Trainer, 1993). Hence PbTxs are voltage dependent Sodium Channel Activators (SCA's) that open and

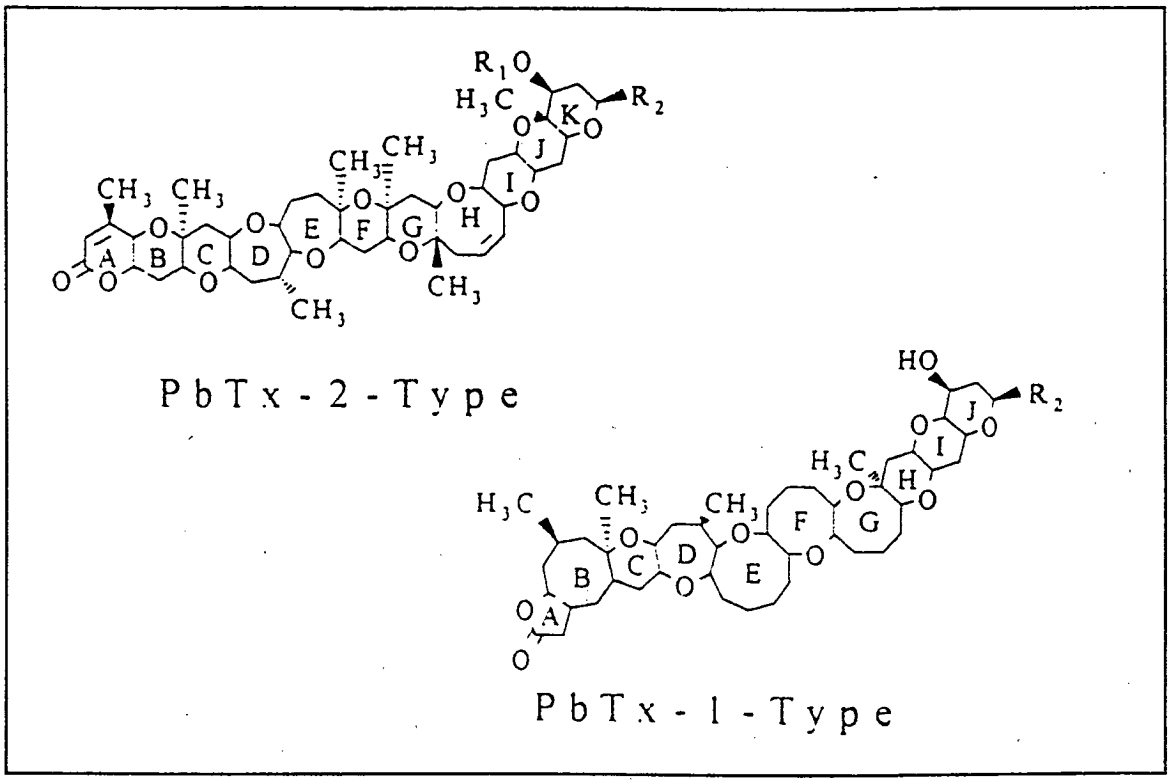


Figure 1.4: Structures of the brevetoxins. The brevetoxins are derived from one of two structural types that are derivatized at R1-R2.

PbTx-2-Type			PbTx-1-Type	
PbTx	**R1**	**R2**	**PbTx**	**R2**
2	H	$CH_2C(=CH_2)CHO$	1	$CH_2C(=CH_2)CHO$
3	H	$CH_2C(=CH_2)CH_2OH$	7	$CH_2C(=CH_2)CH_2OH$
5	CH_3CO	$CH_2C(=CH_2)CHO$	10	$CH_2CH(=CH_3)CH_2OH$
6	H	$CH_2C(=CH_2)CHO$, 27, 28 epoxide		
8	H	$CH_2C(=CH_2)COCH_2Cl$		
9	H	$CH_2CH(CH_3)CH_2OH$		

keep open voltage dependent sodium channels at membrane potentials where they would normally remain closed, leading to contractile paralysis in animal models. PbTxs do not affect potassium currents and their effects are totally counteracted by SCB toxins (Garthwaite *et al.*, 1996, Baden & Trainer, 1993).

The toxic syndrome known as NSP or Neurotoxic Shellfish Poisoning is attributed primarily to PbTxs although ciguatoxins share a common binding site on sodium channels and this may account for similarities in neuropathological symptoms in mammals (Hallegraeff *et al.*, 1995). NSP caused by PbTxs was first found in *Gymnodinium breve* which was thought to be endemic to the Gulf of Mexico and the East coast of Florida where red tides have been reported since as early as 1844. *G. breve* is only one of possibly 2 or 3 gymnodiniod species that produce PbTxs with other raphidophycean flagellates such as *Chattonella marina*, *C. antiqua* (Steidinger *et al.*, 1998), *Fibrocapsa japonica* (Khan *et al.*, 1996) and *Heterosigma akashiwo* (Khan *et al.*, 1997) also reported as producing this class of polyether toxin. Work on multiple clones of *G. breve* (reported as *P. brevis*) by Baden & Thomas (1989) indicated a toxin range per cell of 6.6-16.6pg cell^{-1}.

All PbTx producing representatives have been implicated in fish kills. *G. breve* (reported as *Ptychodiscus brevis*) was implicated in a mass mortality of fish in Florida (Baden & Mende, 1982) while histological studies have shown *C. marina*, *C. antiqua* and *Gymnodinium* sp. to cause severe changes in the gills of yellowtail, *Seriola quinqueradiata* (Doi *et al.*, 1981, Shimada *et al.*, 1982, Endo *et al.*, 1985, Toyoshima *et al.*, 1985). These changes included, severe oedema formation on the secondary lamellae and interlaminar epithelium, swelling and alterations to the gill chloride cells, severely hyperplastic and hypertrophic gill epithelia in red sea bream, *Pagrus major* (Endo *et al.*, 1992), reductions in gill carbonic anhydrase activity, reductions in mucous goblet cells (Shimada *et al.*, 1983) and cardiac irregularities (decrease in heart rate) leading to hypoxia and death from low gill circulation (Endo *et al.*, 1992).

1.5 Phycotoxins in the Marine Food Chain

1.5.1 Zooplankton as a Primary Toxic Vector

Toxic substances produced by dinoflagellates have been shown many times to be propagated through planktonic food webs with potentially lethal effects on their vertebrate components (Boyer *et al.*, 1985, Robineau *et al.*, 1991, Kelly *et al.*, 1992). Herbivorous zooplankton, in one form or another, have been implicated as a primary toxic vector in the marine food web, allowing movement of toxins from phytoplankton to higher trophic levels. (White *et al.*, 1989, Boyer *et al.*, 1985, Yazdandoust, 1985, Gosselin *et al.*, 1989, Hansen, 1989, Kelly *et al.*, 1992). The grazing of zooplankton on phytoplankton is the first step in converting photosynthetically fixed carbon into higher trophic level

biomass. Algal blooms generally represent a large reservoir of labile phytoplanktonic carbon for assimilation by zooplanktonic and crustacean grazers (Sellner & Olson, 1985). Grazing by zooplankton, particularly on toxic phytoplankton, exposes the zooplankton to ingested levels of endogenous algal toxins, although some zooplankton have been observed to actively avoid dense patches of blooming, toxic algae (Fielder, 1982, Huntley, 1982, Sellner & Olson, 1985, DeMott *et al.*, 1991) in the surface waters and lower in the water column. Once ingested the algal toxins may have several different fates such as accumulation in body tissues and possible vectorial transfer, removal as waste or metabolic alteration and transformation (Sullivan *et al.*, 1983, Cembella *et al.*, 1994). Boyer *et al.* (1985) indicated that the copepod *Tigriopus californicus* rapidly accumulated PSP toxins after feeding on *A. catenella* and reached a saturation concentration of 3nmol STX $100mg^{-1}$ dry weight after 14 hours. White (1981) fed wild zooplankton samples (collected from the Bay of Fundy; Canada), dominated by the copepod *Arcatia clausii* and barnacle nauplii *Balanus* sp., with toxic strains of *A. tamarense*. Maximal levels of toxicity were gained within 6 hours with the *Balanus* sp. toxicity being twice (54 µg STX equiv.g^{-1} wet wt.) that observed in the copepod (19-25 µg STX equiv.g^{-1} wet wt.). However, Huntley *et al.* (1982) indicate that the copepods *Calanus pacificus* and *Paracalanus parvus* could not be induced to feed on three strains of toxic dinoflagellate including: *A. tamarense*, *A. tamarense* (Ipswitch), and *Gymnodinium breve*.

Literature concerning the feeding strategies, avoidance responses to toxic algae and discriminatory ability between toxic and non-toxic algal cells, of zooplankton, is abundant. However, some zooplankton do utilize toxic algal cells as a food source and the effects of the endogenous toxins within them has received disproportionately little attention. Some studies of the activity of algal neurotoxins on isolated nerve preparations have demonstrated that invertebrates are (at least, potentially) as susceptible to nerve blockage and paralysis as are vertebrates (Ives, 1985). Hence mortality of zooplankton from ingested algal neurotoxins is possible, although toxicity may simply cause an inability to feed (Ives, 1985). No observations seem to exist as to the toxic effects of endogenous algal hepato-, dermato- or lytic toxins in grazing zooplankton. Fulton & Paerl (1987) reported that there was a strong relationship between the toxicity of *Microcystis aeruginosa* (Cyanophyceae) and its inhibitory effects on herbivore feeding rates, and that the toxins produced by *M. aeruginosa* may, over a long period, cause mortality of the zooplankton. Ciguatera toxin containing dinoflagellates (i.e. *Gambierdiscus toxicus*) proved toxic to *Artemia* sp. with high rates of mortality in *Artemia* at algal cell concentrations of 1000 cells $animal^{-1}$ (Kelly *et al.*, 1992). However, although zooplankton mortality has been observed whilst grazing on toxic algal cells, particular species and strains of algae differ in their degrees of toxicity. Zooplankton taxa also appear to differ in their susceptibilities to these toxins (Rothhaupt, 1991).

Hawser *et al.* (1992) found zooplankton to be resistant to *Trichodesmium* sp. (Cyanophyceae) toxins, particularly copepods, while DeMott *et al.* (1991) found rotifers to be resistant to toxins produced by most cyanobacteria. Carlsson *et al.* (1990) indicate that even at relatively low densities of toxic algal cells, zooplankton mortality can still occur when the algal strain itself has sufficient quantity or potency of toxin. Carlsson *et al.* (1990) describe the growth and ingestion rates of *Favella ehrenbergii* (tintinnid ciliate) as being negatively affected by the addition of *Chrysochromulina polylepsis* (Prymnesiophyceae) with death rates in *F. ehrenbergii* being the same as when they where starved in seawater without food. Hence zooplankton mortality can occur from toxic algal cell ingestion, depending on the toxic potency of the ingested cell. However, some zooplankton seem resistant to particular algal toxins.

Herbivorous zooplankton have been found to be able to accumulate toxins, particularly the PSP toxins (White, 1980, White, 1981, Hayashi *et al.*, 1982, Boyer *et al.*, 1985, Robineau *et al.*, 1991). Toxin content of the zooplankton has been found to approximately parallel the number of toxic algal cells within the water (White, 1979, Hayashi *et al.*, 1982). Zooplankton have also been observed to retain algal toxins for some degree of time. Hayashi *et al.* (Hayashi *et al.*, 1982) indicate that toxins were acquired and retained for several days post ingestion, whereas Kelly *et al.* (1992) observed toxic *Gambierdiscus toxicus* cells remaining in the gut of *Artemia* for several hours post ingestion. Hence, in digestive periods, predated zooplankters would act as toxic vectors. Robineau *et al.* (1991) disagrees with this and states that zooplankton are unable to accumulate toxins which would limit their ability to propagate algal toxins through the food web.

The primary result of toxins ingested by zooplankton is a modified behaviour pattern (which may lead to mortality), particularly if only a toxic, sub-lethal threshold can be reached (due to reduced feeding rates: Boyer *et al.*, 1985), this may then increase the likelihood of the vectorial passage of toxins to finfish, particularly as finfish that feed on herbivorous zooplankton, are strictly visual hunters (Eggers, 1977, Heller & Milinski, 1979, O'Brien, 1987), with unusual behaviour of prey being of prime importance (Eggers, 1977, O'Brien, 1987). These individuals are more likely to be identified and consumed by finfish, thereby completing the zooplankton's role as a toxic vector. This type of toxin passage could theoretically occur up the food chain to the very highest levels.

1.5.2 Phycotoxins Transferred to Higher Trophic Vertebrates

Prior to knowledge of fish-kills, their causes and dynamics, it was presumed that finfish, like other

cold blooded vertebrates, were relatively insensitive to algal toxins. However, this was in a period when only preliminary research had been conducted on fish kills due to PSP toxin producing algae. Other phycotoxins at this time were largely undocumented. It is now well known that finfish can be highly susceptible to phycotoxins (Mortensen, 1985, Adnan, 1989, Toyoshima *et al.*, 1989, Chang *et al.*, 1990). PSP toxins (most intensively studied of all phycotoxins) have been shown to be lethal to finfish in low oral doses and extremely low intraperitoneal (i.p.) doses (White, 1981). The same symptoms for oral and i.p. doses, including loss of equilibrium within 5-15 minutes followed by immobilization and shallow arrythmic breathing were observed, with death usually occurring 20-60 minutes after toxin administration, regardless of the route (White, 1981, White, 1984).

The majority of finfish cannot tolerate even small systemic concentrations of algal toxins (PSP toxins) and thus they are unable to accumulate them. This is of particular significance in commercial finfish industries as dead fish are not taken for human consumption. Finfish toxicity is also confined to the viscera, with the meat remaining toxin-free (White, 1981, White, 1984). This seems to represent an advantage to human consumers, where dead fish are not taken, and with only the meat being consumed; however, it does not necessarily exclude higher trophic components (such as marine mammals) from the effects of toxic algae.

Marine mammals represent the highest trophic components in the marine algal food chain. Mortalities of marine mammals associated with algal blooms have been documented many times (Gunter *et al.*, 1947, Anderson & White, 1989, Geraci *et al.*, 1989, Joyce, 1989) as has the threat to, or influence of, the feeding behaviour and distribution in high level marine consumers (eg. whales, dolphins, porpoises, seals, seabirds)(Kviteck *et al.*, 1991). Vectorial passage of toxins via finfish, such as Atlantic mackerel, has been shown to be the primary route of intoxication. This intoxication is particularly seen in the Odontocetes and possibly Pinnipeds but also specific species such as the Humpback whale, *Megaptera novaeangliae* (Geraci *et al.*, 1989). Toxic marine zooplankton, however, may pose a more general threat to the planktonic feeding Baleenoptera.

Unlike human and other terrestrial consumers that have broad ranging sensitivities to phycotoxins, the lethal levels for aquatic mammals may be even less, as they must orientate themselves properly to breath (Anderson & White, 1989). Hence, even moderate symptoms may lead to drowning. This fact is compounded by the mammalian diving reflex (in those consumers that dive) which is highly developed in marine mammals. This reflex causes the shunting of blood, when the mammal dives, away from peripheral tissues such as gut, kidneys and liver (the visceral organs where algal toxins are usually

concentrated, detoxified and excreted) to the heart and brain. Hence any algal toxins, specifically neurotoxins, that are present are moved to areas where their presence would be the most acute.

1.6 The Toxic Dinoflagellates *Alexandrium minutum* and *Gymnodinium catenatum* - taxonomy

1.6.1 *A. minutum* Halim 1960

The cells of *A. minutum* Halim (Fig 1.5) are subspherical and ovoid in shape. They occur singly or rarely in pairs in both culture and field samples alike. The cells are typically small, 22-26μm long and 19-23μm wide (Hallegraeff *et al.*, 1991). Both the cingulum and sulcus are deeply excavated, with the cingulum being displaced from 1 to 1.5 times its width while the sulcus extends to the antapex. The first apical plate has a small ventral pore on its right anterior margin and is often in direct contact with the apical pore complex. The sixth precingular plate is narrow. The anterior sulcal plate is approximately as long as it is wide with a straight or almost straight anterior margin, while the posterior sulcal plate is almost always symmetrical. The only variable feature is the peculiar hypothecal sculpture that is found in Italy, reduced in Egypt and Australia (in Western Australia but not South Australia), but is essentially the same pattern in all of them (Balech, 1995).

A. minutum produces, as part of its life cycle, a colourless mucoid resting cyst approximately hemispherical in shape but circular (24-29μm diameter) when seen from above and reniform (15-19μm high) when observed in lateral view (Hallegraeff *et al.*, 1991). The alga produces dense, annually recurrent blooms in the Port River area near metropolitan Adelaide, South Australia in the period September - November (Cannon, 1990) with cellular concentrations observed of up to 10^8 cells L^{-1}. The current distribution of *A. minutum* ranges from the whole of the Mediterranean Sea, Atlantic coasts of Spain, Portugal (Galician coastal waters) and France (Vilaine Bay, Brittany), Irish Sea, Indonesia, southern Taiwan (Chiku, Tainan county), East coast of the USA (Mineola in New York State), southern Australia (Port River near Adelaide, Newcastle, Perth), Philippeans, Japan, Thailand and New Zealand (Bay of Plenty, Marlborough Sound).

The strain of *A. minutum* used in these studies (AMAD-06) was isolated (J. Cannon/S. Blackburn) on 27th October 1987 in the Port River, Adelaide region of Australia.

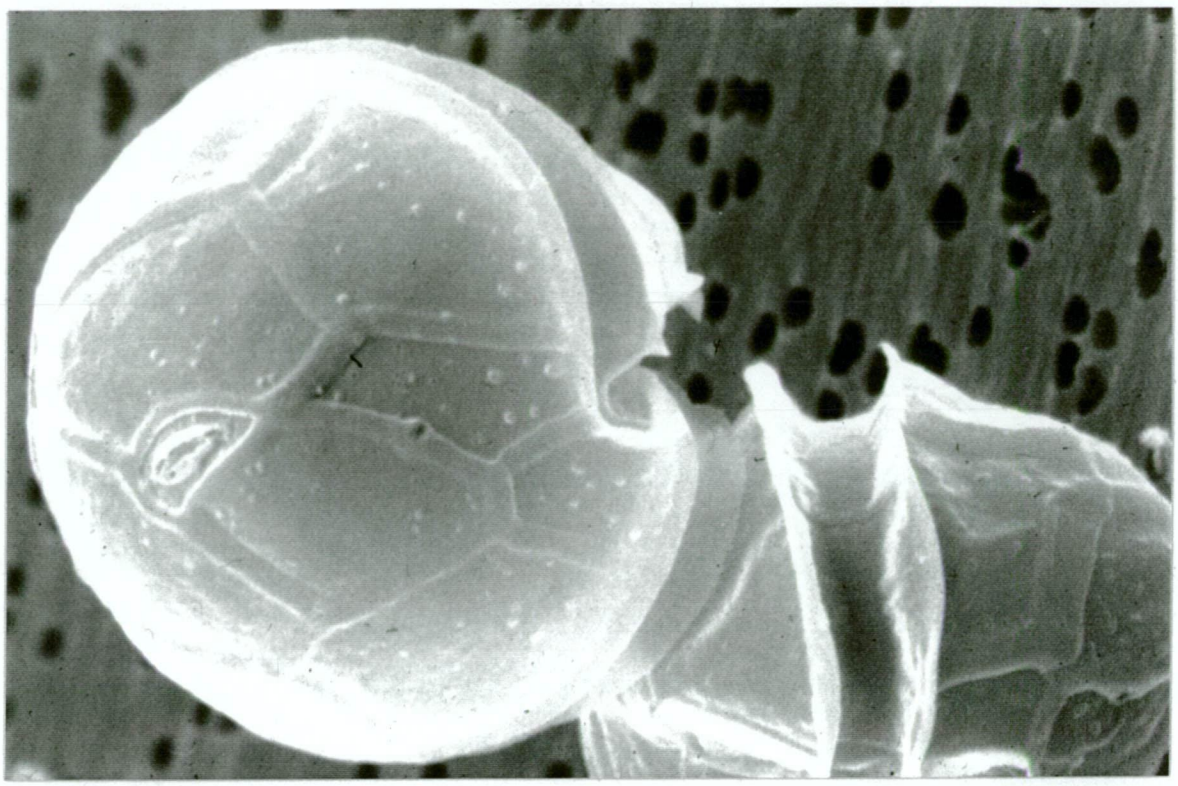


Figure 1.5: The armoured, PSP (neurotoxic) producing dinoflagellate, *Alexandrium minutum* (Halim) (from: Hallegraeff 1991). SEM x 4300.

1.6.2 *G. catenatum* Graham 1943

The unarmoured, chain forming dinoflagellate *Gymnodinium catenatum* was first described from the Gulf of California by Graham over 50 years ago (Graham, 1943) (see Fig. 1.6). The dinoflagellate produces snake-like chains commonly comprising 4, 8, 16 or 32 but sometimes as many 64 cells in length, however stationary phase cultures contain predominantly single cells (Blackburn *et al.*, 1989). The cells themselves vary greatly in size and shape (subspherical, biconical or squarish) but are generally 23-41μm long and 27-36μm wide with a covering of roughly hexagonal amphiesmal vesicles. The girdle groove is deep with a left handed displacement, over 1/5th to 1/3rd of the cell length and the sulcus is deep and extends well into both the epicone and the hypocone (Blackburn *et al.*, 1989, Hallegraeff, 1991). The apex of the cell has a horse shoe shaped apical groove to the left and dorsal of the anterior attachment pore that connects the cells in a chain with unfavourable conditions causing the chains to break up at these points into single cells. During extended periods of unfavourable conditions *G. catenatum* can form both resting cells (40-44μm long, 27-31μm wide) and temporary cysts (35-40μm diameter, spherical bodies) (Blackburn *et al.*, 1989) with the

brown, spherical, benthic temporary cyst having microreticulate surface markings that reflect the pattern of the amphiesmal vesicles (Hallegraeff, 1991). The cysts of *G. catenatum* can germinate rapidly (within two weeks of encystment) under a wide range of environmental conditions (Hallegraeff *et al.*, 1995). The current world distribution of *G. catenatum* includes Japan, Portugal, Italy, Australia and Venezuela.

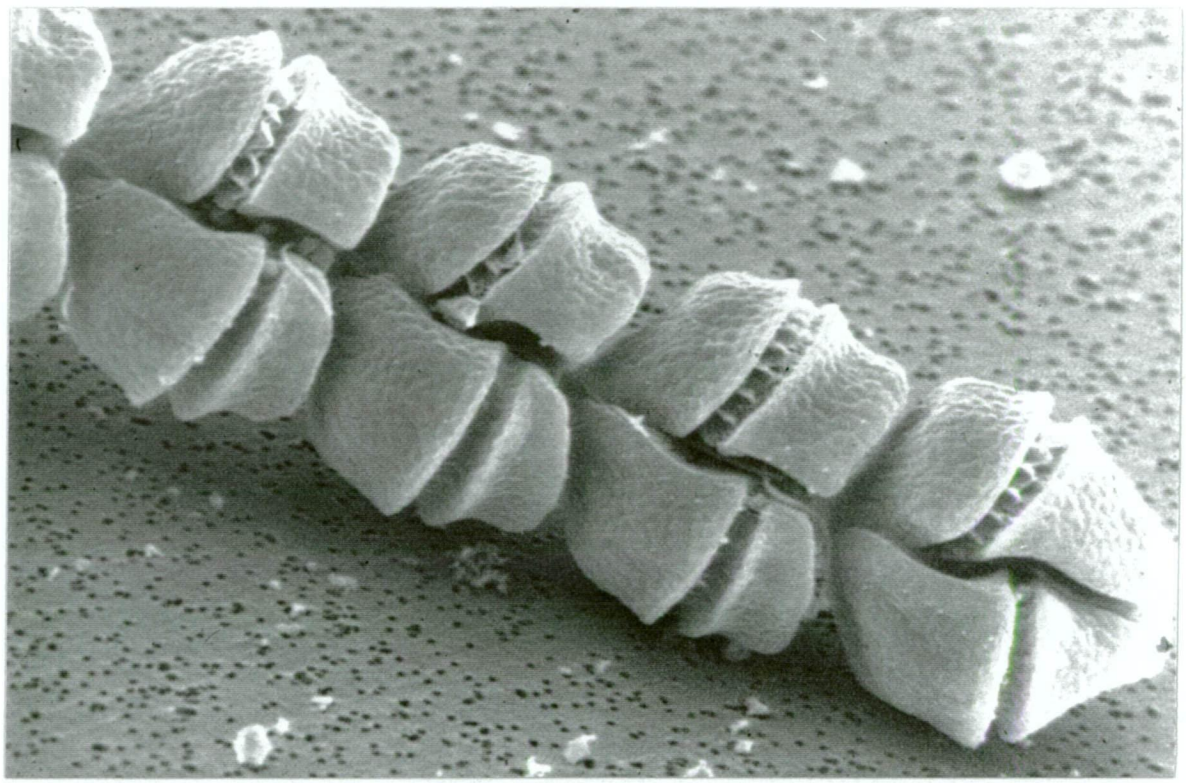


Figure 1.6: The unarmoured, chain-forming, PSP (neurotoxic) dinoflagellate *Gymnodinium catenatum* (Graham) (from: Hallegraeff 1991). SEM x 1400.

The strain of *G. catenatum* used in these studies (GCDE-08) was isolated (S. Blackburn) as a chain of eight cells from the Derwent River Estuary (Tasmania, Australia) on the 15th June 1987 as one of nine clones isolated between January 1986 and July 1987 (Oshima *et al.*, 1993). Based on an analysis of historic plankton samples and cysts in sediment depth cores, it is believed this species is not indigenous to Tasmania but introduced into the region after 1973 (Hallegraeff *et al.*, 1995).

1.7 Research Focus

The following research stems from previous studies conducted in our laboratory investigating

the toxicity of two strains of Australian toxic dinoflagellates (*A. minutum*, *G. catenatum*) and one toxic raphidophyte (*Heterosigma akashiwo*) to experimental fish (*Poecilia reticulata*) and zooplankton (the brine shrimp *Artemia salina* and rotifer *Brachionus plicatilus*) (Lush, 1993). This research is presented as Chapter 2 to provide background for the main body of the thesis. This research indicated: 1) that *G. catenatum* was the possible cause of of the CNG syndrome appearing in Tasmanian farmed Atlantic salmon concurrent with blooms of this dinoflagellate, and 2) that the exocellular growth medium of *A. minutum* appeared to be highly toxic to brine shrimp, indicating an unknown toxic phenomenon (Lush & Hallegraeff, 1996).

It was decided to primarily investigate the link between *G. catenatum* and the CNG syndrome in Atlantic salmon (Chapter 3) as this was a problem that was current, virtually unresearched, local, and of great importance to the regional aquaculture industry in terms of maintaining healthy and disease free fish stocks. However, it appeared that the initial experimental protocol of bioassays exposing fish to pure cultures of *G. catenatum* severed the link between *G. catenatum* itself and the CNG syndrome. Hence, we decided to concentrate on *A. minutum* and its apparently toxic exocellular medium. This primarily took the form of bioassays (Chapter 4) using pure cultures of a strain of *A. minutum* (AMAD-06) sourced in the Port River region of Adelaide, Australia, against firstly *A. salina* (death only) and secondly the flounder *Rhombosolea taparina* (histopathology and clinical pathology). The bioasays consisted of exposures to pure cultures, filtered culture medium and crude lipophilic extracts of the dinoflagellate with resultant documentation of death, behavioural changes, pathological tissue change and clinical pathology purtebations from both acute and chronic exposures. The documentation of the effects of *A. minutum* cultures was then followed by an intensive investigation of the toxicity of the dinoflagellate over its entire life cycle (Chapter 5) including total toxin content and toxin composition of the cultures endocellular fraction and the toxicity of the exocellular fraction (culture medium) using both *A. salina* bioassays and the relatively new neuroreceptor binding assays developed by G. Doucette (USA) and the tetrazolium-based neuroblastoma bioassay of I. Garthwaite (New Zealand). Assays such as these that allow the analysis of toxins disolved in seawater are part of a relatively new field and as such literature on the subject as scarce as is research. A control experiment was also undertaken to document the role (if any) of bacteria in the apparent exotoxicity of *A. minutum* (Chapter 6). All errors presented with means, in the thesis, are standard deviations with the number of replicates used to calculate those means and errors presented immediately afterward in brackets.

Chapter 2: Exotoxicity in two Australian species of PSP dinoflagellates, *Alexandrium minutum* and *Gymnodinium catenatum*: Preliminary observations[1].

2.1 Introduction

The production and release of non-toxic exudates by phytoplankton is well documented (Gentien & Arzul, 1990) as is the release of toxic exudates by some members of the Prymnesiophyceae (*Chrysochromulina, Prymnesium*) (Gomes *et al.*, 1991) and possibly the Raphidophyceae (*Chattonella, Heterosigma*). However, little is known of active toxin excretion into the water column by toxic species of the Dinophyceae (*Alexandrium, Gymnodinium, Pyrodinium*) that may affect zooplankton (Edvardsen, 1993; Ogata & Kodama, 1986), fish or higher organisms such as marine mammals. Zooplankton appear to display wide interspecific differences in their vulnerability to toxic phytoplankton but larvae of some copepod species seem to share the resistance of their adult counterparts (Simonsen *et al.*, 1995). In the case of fish, it has been suggested that first feeding larval fish would need to ingest less than 10 cells of only a moderately toxic dinoflagellate (*A. tamarense*) for death to occur (Robineau *et al.*, 1991). Anecdotal links have also been made between toxic phytoplankton blooms and the death of whales (Geraci *et al.*, 1989) and seals (Anderson, 1997). The effects of toxic phytoplankton cells and their exudates on zooplankton and fish thus could have severe implications for aquaculture and the marine environment in areas prone to recurrent toxic phytoplankton blooms. This is particularly so of areas prone to blooms of phytoplankton species that produce exocellular toxins. Threats to human recreational users of waterways contaminated with toxic algal exudates, particularly the possibility of PSP exudates, are unknown, but may be great considering the human sensitivity to low oral doses of saxitoxin (6.5-16.0 μgKg-1, Evans, 1972).

This chapter, in the light of recent research (Lush, 1993, Lush & Hallegraeff, 1996), reports on a novel exotoxic principle from the dinoflagellate *A. minutum*, that may have sodium channel binding properties, and is capable of killing the brine shrimp *Artemia salina*. This

[1] An earlier version was published as: G. J. Lush and G. M. Hallegraeff, High Toxicity of the Red Tide Dinoflagellate *Alexandrium minutum* to the Brine Shrimp *Artemia salina*, In: Yasumoto, T., Oshima, Y. and Y. Fukuyo (Eds), *Harmful and Toxic Algal Blooms*, IOC of UNESCO, p. 389-392, (1996)

exotoxic effect is compared with another PSP dinoflagellate, *Gymnodinium catenatum* (Oshima *et al.*, 1987), and the known haemolytic raphidophycean flagellate *Heterosigma akashiwo*.(Chang *et al.*, 1990) The non-toxic green flagellate *Tetraselmis suecica* was used as a control.

2.2 Materials and Methods

2.2.1 Algal cultures

Cultures of the toxic phytoplankton species were obtained from the CSIRO Marine Laboratories, Hobart (Table 2.1). Phytoplankton where grown to the early stationary phase (>10^6 cells L^{-1}), under 50μE m^{-2} s^{-1} light, 12hr/12hr L:D and 17°C temperature in either GP (*Heterosigma*), GSe (*Gymnodinium, Alexandrium*) or f medium (*Tetraselmis*) (Appendix 1). *Artemia* were hatched and grown as in Appendix 2. All experimental procedures were replicated three times.

Table 2.1: Culture materials used in this study; origins and designated codes.

Species	Culture Code	Origin	Toxicity
Dinoflagellates			
Alexandrium minutum Halim	AMAD-06	Port River, South Australia	PSP
Gymnodinium catenatum Graham	GCDE-08	Derwent River, Tas., Australia	PSP
Raphidophyte			
Heterosigma akashiwo Hada	CS-169	West Lakes, South Australia	Hemolytic, Ichthyotoxic
Prasinophyte			
Tetraselmis suecica (Kylin) Butcher	CS-187	Brest, France	Non-toxic, Control

2.2.2 Effects on grazing

Four 40 mL subsamples of pure phytoplankton culture from each of the four phytoplankton

species were placed separately in 100 mL Schött bottles. One hundred 4-day old *Artemia* metanauplii were introduced into each bottle except in the non-grazed controls. All bottles were then placed in a 12hr/12hr L:D, 20°C controlled temperature cabinet for 10 days. Bottles were gently swirled three times a day to maintain an even distribution of phytoplankton cells. All bottles were subsampled daily for cell counting and estimates of *Artemia* mortality.

2.2.3 Exudate affects

Subsamples (10mL) of each phytoplankton culture were gently gravity screened through 5µm plankton mesh and then filtered through a 0.45µm filter membrane (Schleicher & Schüll; Membranfilter ME 25). This culture-filtrate was placed in 50 mL Schött bottles with 5mL of *T. suecica* culture. Bottles were then placed in a 23.5°C waterbath for 1hr prior to the addition of *Artemia*. One hundred 4-day old *Artemia* metanauplii were placed in each of the experimental bottles. Phytoplankton cell numbers were counted over time (18 and 24h) and compared to an initial count. Bottles were periodically checked for *Artemia* mortality. Controls included *T. suecica* cell-free culture media with *T. suecica* whole cells and the above protocol repeated without *Artemia*.

2.2.4 Mortality due to whole-cell cultures and culture-filtrate

Artemia bioassays were carried out as Appendix 2. Trials were conducted with each of the three toxic phytoplankton species and the non-toxic control initially with whole cell cultures and then culture-filtrate if mortality was observed in the whole cell trials. Trials with pure GTX_1-GTX_4 toxins (CNRC CANADIAN TOXINS) were also carried out. GTX toxins were dissolved in seawater, after acidic conversion (100°C for 5min with 0.1M HCL) from C_1-C_4 fractions, to a concentration to match that found in the *A. minutum* whole cells culture medium based on 1pg STX eq. $cell^{-1}$ (C. Soames: personal communication) assuming up to 10% of leakage of PSP toxins from senescent cells (A. Cembella, Y. Oshima, personal communication) as this is the only process thought to enable PSP toxins to enter the surrounding seawater (Onodera *et al.*, 1996). Ten *Artemia* from one of three different life cycle stages (first-day nauplii, 4-day metanauplii or adults) were placed in each exposure well. All exposure plates were held at a constant 20°C in darkness. Sequential wells were examined each hour for *Artemia* mortality over a 24 hr period.

Samples of *A. minutum* and *G. catenatum* culture-filtrate were also analysed for sodium channel blocking (SCB) activity (Courtesy G. Doucette, Marine Biotoxins Program, National Marine Fisheries Service, South Carolina, USA) using a site 1 specific, neuroreceptor binding assay (see section 5.2.3.1). Although this assay does not conclusively prove that PSP toxins are present it does indicate that PSP-like toxins are present in the form of SCB substances with an activity similar to that exhibited by standard saxitoxin. A definitive assay for PSP toxins in seawater is, at present, only in the developmental stage (electrochemical assay by G. Boyer).

2.3 Results

2.3.1 Effects on grazing

Artemia metanauplii fed at a high rate of approximately 356 ± 8.0 cells min^{-1} $animal^{-1}$ on the non-toxic *T. suecica* while in the absence of *Artemia* phytoplankton concentration steadily increased at an average rate of 7.8 x 10^4 cells $min^{-1}L^{-1}$ (Table 2.2). In all grazing trials with toxic phytoplankton 100% *Artemia* mortality was eventually observed.

Table 2.2: Growth rates of *T. suecica* culture in the presence of filtered growth medium of *A. minutum*, *G. catenatum*, *H. akashiwo* and *T. suecica* only (controls) and with the addition of *Artemia salina* as grazers.

Alga	Control Culture Initial cell conc. (x10^7/L)	Algal growth rate (cells/min)	Grazing Experiment Initial cell conc. (x10^7/L)	Mean grazing rate (cells/min/animal)
Alexandrium minutum	6.7 ± 1.4	5473 ±1711	6.5 ± 0.5	-19.3 ±6.4
Gymnodinium catenatum	0.03 ± 0.0	-74.8 ±14.5	0.3 ± 0.0	9.4 ±0.4
Heterosigma akashiwo	12.3 ± 2.2	6845 ±1175	22.5 ±1.8	74.8 ±8.1
Tetraselmis suecica	26.7 ± 2.5	78392 ±3086	37.5 ± 2.6	356 ±8

When exposed to *H. akashiwo*, *Artemia* fed at a lower average rate (74.8 ± 8.1 cells min^{-1} $animal^{-1}$) than in the control. Feeding by *Artemia* on the PSP dinoflagellate *G. catenatum* was

further reduced (9.4 ± 0.4 cells min^{-1} animal-1) while *Artemia* feeding on *A. minutum* was negligible, with an apparent increase in cell numbers (-19.25 ± 6.4 cells min^{-1} animal-1). *Artemia* mortality in the PSP dinoflagellate trials occurred within 24h with behavioural changes prior to death including erratic swimming, immobility, clumping of uncoordinated individuals. *Artemia* exposed to *H. akashiwo* displayed similar symptoms but only after a longer incubation period (70-100h) with average mortality occurring within 82 ± 14.5h.

2.3.2 Culture-filtrate effects on feeding

The growth of *T. suecica* cultures was not affected by cell-free media from the three toxic phytoplankton nor by its own culture medium. No significant reduction in the number of *T. suecica* cells over the 24 hr test period was observed in any of the control flasks, hence changes in *T. suecica* cell numbers in the experimental trials were due to grazing by *Artemia.*

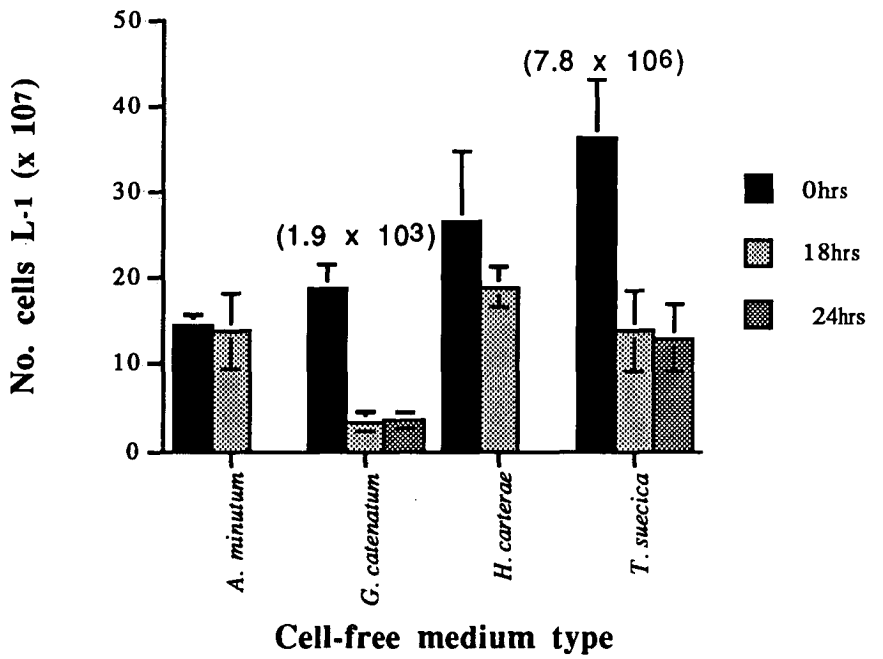


Fig 2.1: Feeding response of 4-day old *Artemia* metanauplii on non-toxic cells of *T. suecica,* in the presence of cell-free culture media from the three toxic phytoplankton species, *A. minutum, G. catenatum* and *H. akashiwo.* () = mean rates of feeding (cells $min.^{-1}$ $animal^{-1}$).

A significant reduction in the number of *T. suecica* cells occurred after 18h in the bottles

containing *Artemia* and *T. suecica* culture-filtrate (approximately 62% decrease), and *Artemia* and *G. catenatum* culture-filtrate (82% decrease) (Fig. 2.1). In both experiments, no *Artemia* mortality was observed within the 24hr experimental period. In contrast, no statistically significant reduction was observed in *T. suecica* feed cells in bottles containing *Artemia* and *A. minutum* or *H. akashiwo* culture-filtrate. In both *A. minutum* and *H. akashiwo* experimental bottles 100% mortality of *Artemia* occurred within 24h.

2.3.3 Mortality due to whole-cell cultures and culture-filtrate

Differences in survival of the three life cycle stages of *Artemia* were observed upon exposure to the four phytoplankton species (Table 2.3). No mortality was observed in trials with newly hatched (<24h) nauplii larvae, probably due to their reliance on yolk sacs for nutrition where consumption produced mortality but more importantly due to branquipods being more developed in the adult stage with the branquia being mostly affected by hemolytic substances.

Table 2.3: Time to death of 50% of *Artemia* (three different life cycle stages) on exposure to whole cell and cell-free cultures of the toxic algal species, *A. minutum*, *G. catenatum* and *H. akashiwo*. Non-toxic *T. suecica* was used as a control.

Alga	*Artemia* larval stage	tD50 (hrs) whole culture	tD50 (hrs) cell-free culture
A. minutum	nauplii	n.m.	n.a.
	metanauplii	10.9±1	5.8±0.6
	adult	16.8±0.4	7.7±0.8
G. catenatum	nauplii	n.m.	n.a.
	metanauplii	18.4±0.3	no mort.
	adult	n.m.	n.a.
H. akashiwo	nauplii	n.m.	n.a.
	metanauplii	20.4±1.7	37.6±5.6
	adult	n.m.	n.a.
T. suecica	nauplii	n.m.	n.a.
	metanauplii	n.m.	n.a.
	adult	n.m.	n.a.

n.m. = no mortality observed

n.a. = not applicable (no mortality observed from whole-cell culture)

Exposure to *H. akashiwo* resulted in mortality of the metanauplii stage only (tD50 = 20.37 ± 4.80h) while cell-free *H. akashiwo* culture medium also resulted in *Artemia* mortality (37.6 ± 5.6h). Adult *Artemia* exposed to *H. akashiwo* showed uncoordinated and reduced swimming activity with long periods of total inactivity, although no mortality was observed over the 24hr period. All metanauplii and adult *Artemia* exposed to *A. minutum* died within the experimental period when exposed to whole cells (tD50=10.91 ± 3.00h) or culture-filtrate (tD50= 5.84±0.58h). The metanauplii were the most sensitive stage, with the culture-filtrate producing the fastest mortality response. The adults were somewhat more tolerant of *A. minutum*, and again the culture-filtrate elicited the most rapid response (tD50 = 7.72 ± 0.74h; compared to the whole cells exposure, tD50 = 16.78 ± 0.43h). By comparison, *Artemia* metanauplii exposed to pure GTX $_{1\text{-}4}$ showed no significant mortality within a 24 hr period. Only *Artemia* metanauplii showed 100% mortality (tD 50 = 18.39 ± 0.98h) when exposed to whole cells of *G. catenatum* but no response was observed in metanauplii or adults exposed to cell-free *G. catenatum* culture medium.

The culture-filtrate of *A. minutum* tested positive for the presence of a Sodium Channel Blocking (SCB) substance(s) equivalent to 97nM of saxitoxin. The cellular concentration of *A. minutum* in the culture before the cells were removed was $1.72 \times 10^7 \pm 1.19 \times 10^6$.and using an endocellular toxin concentration of 1.0 pg STX equiv. cell^{-1} (C. Soames 1996, pers. comm.) we can calculate an endocellular toxin concentration of 46.17 ± 3.19nM STX equivalents which represents approximately one third of the total SCB pool (~143nM STX equiv.) in the original culture. This confirms unpublished data of E. Maas (1996, personal communication) who found by ELISA, 67% of the total "PSP" toxin pool in the exocellular medium of *A. minutum* with the remaining 33% found within the phytoplankton cells. The culture-filtrate of *G. catenatum* tested negative for a SCB toxin.

2.4 Discussion

The present results demonstrate a significant lethal effect by both whole cells and cell-free culture media of *A. minutum* on the metanauplii and adults of *Artemia*. The metanauplii were the most sensitive stage confirming studies by Demaret *et al.* (1995). Although whole cells of *G. catenatum* also produced a lethal effect in the *Artemia*, its cell-free culture media did not. Such zooplankton mortality from cell-free culture media has not been reported previously in the

presence of *A. minutum*. Since *Artemia* did not feed significantly on *A. minutum*, it is concluded that the high toxic potential by this dinoflagellate is caused by exocellular toxins in the culture medium. The death of *Artemia* from the whole cells and cell-free culture media of *A. minutum* would seem to be due to a fast acting toxin distinct from PSP toxins as pure C_{1-4} fractions (acid converted to GTX_{1-4}) produced no mortality comparable with that in the whole cells or cell-free experiments. However, the presence of a SCB in high concentrations suggests either that there is more than one toxic principle present or a single toxin, chemically distinct from PSP toxins is present with SCB properties (e.g. tetrodotoxin). The difference in structure could account for the different *Artemia* death times but the same pharmacological action.

Dinoflagellates are not generally known to excrete PSP toxins into their surrounding medium (Ogata & Kodama, 1986) but the sodium channel binding activity found in the *A. minutum* culture-filtrate would seem to provide strong evidence that toxins of a PSP nature are being exuded from the cells of this phytoplankton. This would not seem to be an artifact of filtering and cell removal as *G. catenatum* was treated in a similar way to remove the cells and no SCB substance was detected in the exocellular medium by neuroreceptor binding assay. Similarly, the SCB substance is not thought to be a result of "leaky" senescent cells as the cultures used were healthy and in the early stationary phase.

The following research was based on the observations made in this previous work. Research was firstly based on disproving the exotoxic effect of *G. catenatum* as implicated in the CNG syndrome of farmed Atlantic salmon (*Salmo salar*). After this the research focus was shifted to proving the existance of a novel exotoxic principle in *A. minutum* culture medium and documenting its effects on marine fish. Once this was achieved the exotoxic principle of *A. minutum* was followed throughout the life cycle of this phytoplankton with attempts at identifying several of its characteristics and also to determine its specific origin.

Chapter 3: *Gymnodinium catenatum* does not induce gill damage in flounder (*Rhombosolea taparina*)

3.1 Introduction

The Huon River and D'Entrecasteaux Channel in southern Tasmania, Australia (Fig. 3.1), are areas of intensive salmonid and mussel aquaculture. The region is also subject to regular blooms of the toxic dinoflagellate *Gymnodinium catenatum* (Fig. 3.1) and have been subjected to such blooms since they were first observed in 1980 (D. Thomas; unpublished observation: 1980). Consumption of *G. catenatum* cells by mussels in the midst of these blooms and subsequent bioaccumulation of the PSP toxins within them have, in the past, produced some of the highest levels of PSP toxins in shellfish ever recorded in Australia [17 000µg STX equiv.$100g^{-1}$ shellfish meat in longline mussels, Desolation Bay, Tasmania; Hallegraeff *et al.* (1995)]. Such high toxicity of harvested mussels regularly leads to closures of affected mussel farms based on an Australian quarantine level of 80µg STX equiv.$100g^{-1}$ shellfish meat. However, the mussels themselves seem unaffected by such high PSP toxin levels.

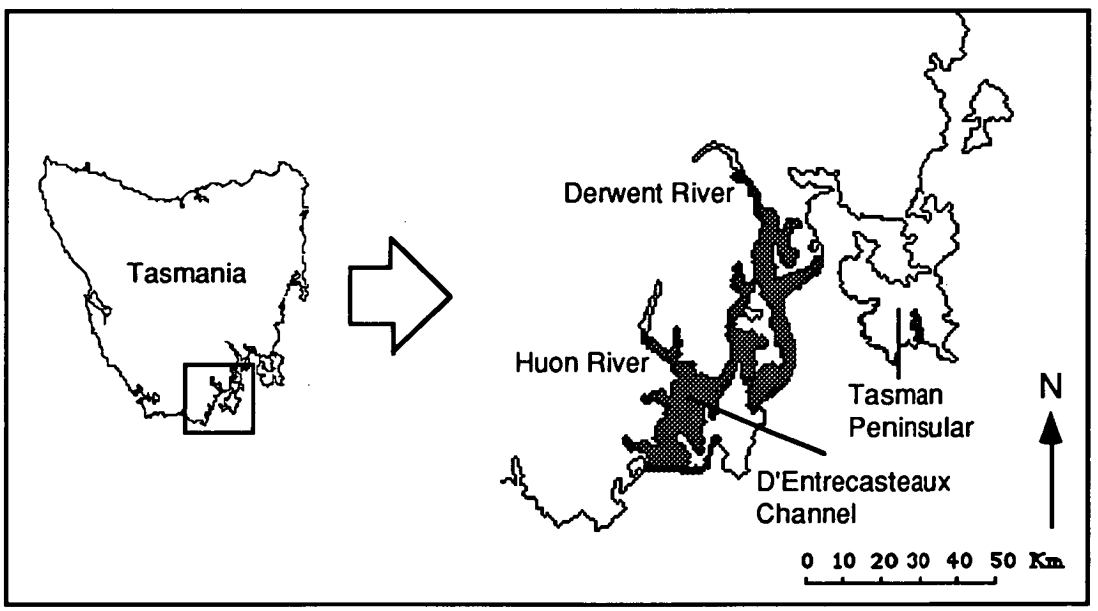


Figure 3.1: Map of Tasmania (Australia) and southeastern Tasmania showing the Huon and Derwent Rivers, D'Entrecasteaux Channel and the Tasman Peninsula. Shaded area indicates regions subject to blooms of the toxic dinoflagellate *Gymnodinium catenatum.*

Salmonids in the area were also thought to be unaffected by the *G. catenatum* blooms,

particularly as *G. catenatum* was not known to produce any exocellular toxins and farmed salmonids are fed on exogenously supplied food pellets and not natural phytoplankton.

Other members of the genus *Gymnodinium* have been found to produce lipophilic, cytotoxic substances (Onoue *et al.* 1985, Partensky *et al.* 1989, Bodennec *et al.* 1993, Mackenzie *et al.* 1995). Jones *et al.* (1982) and Roberts *et al.* (1983) reported severe necrosis and sloughing of the epithelial tissues of the gut and gill in salmon when exposed to *G. mikimotoi* (reported as *Gyrodinium aureolum*), similarly Onoue & Nozawa (1989) found gill damage (oedema and haemorrhage) in juvenile red seabream when exposed to hemolytic and hemagglutinitive fractions of *G. pulchellum* (reported as *Gymnodinium* type '84K) a species similar to that found in southern Tasmania (Hallegraeff 1986: pers. comm.). Nielsen (1993) reported pathological changes in juvenile cod (*Gadus morhua*) from exposure to *G. galatheanum* while Kajiwara *et al.* (1992) observed a cell-destroying sesquiterpenoid from *G. mikimotoi* (reported as *G. nagasakiense*) that was actually excreted from the phytoplankton into the surrounding media.

G. catenatum (strain GCDE-08) is not known to produce any toxins other than its normal compliment of endogenous, low potency N-sulfocarbamoyl (PSP) toxins, the bulk (up to 99 mole%, Oshima *et al.* 1987; Oshima *et al.* 1990) of which comprise C-fractions and minor amounts of the decarbamoyl toxins (Oshima *et al.* 1993) (see Chapter 1). Similarly, it has never been reported to have any ichthyotoxic ability nor produce pathological gill lesions. However, in the autumn of 1993, concurrent with a large *G. catenatum* bloom, the gills of sea-caged Atlantic salmon (*Salmo salar*) smolts were highly necrotic and covered in a thick mucous (Fig. 3.2).

Histologically, the lesions appeared as focal, sometimes full thickness, gill necrosis which in the initial stages showed no host reaction (Clark *et al.* 1997). The healing of these damaged areas gave rise to conspicuous expanded tips or "clubbed" gill filaments with lamellae fusion proximally along the filament with vascular dilation also being apparent (Clark *et al.* 1997). The term clubbing and necrosis gill (CNG) syndrome has been coined to describe the condition. Behaviourally the fish became sluggish, showed a poor feeding response and extended periods of surface swimming. Fish left in their cages without handling stress showed no signs of respiratory stress. The gill pathology and behavioural changes coincided with the

massive 1993 bloom of *G. catenatum* (Hallegraeff *et al.* 1995). It also became apparent that such pathological changes in fish gills had occurred previously (1988, 1990, 1991, 1992), concurrent with *G. catenatum* blooms, and that the effect was not confined to Atlantic salmon but also present in wild Huon River flounder and cultured trout (J. Handlinger: pers. comm.) and have also been seen in a Murray cod (B. Munday: pers. comm.). Thus while spectacular, the lesions are not necessarily related to a single aetiological factor.

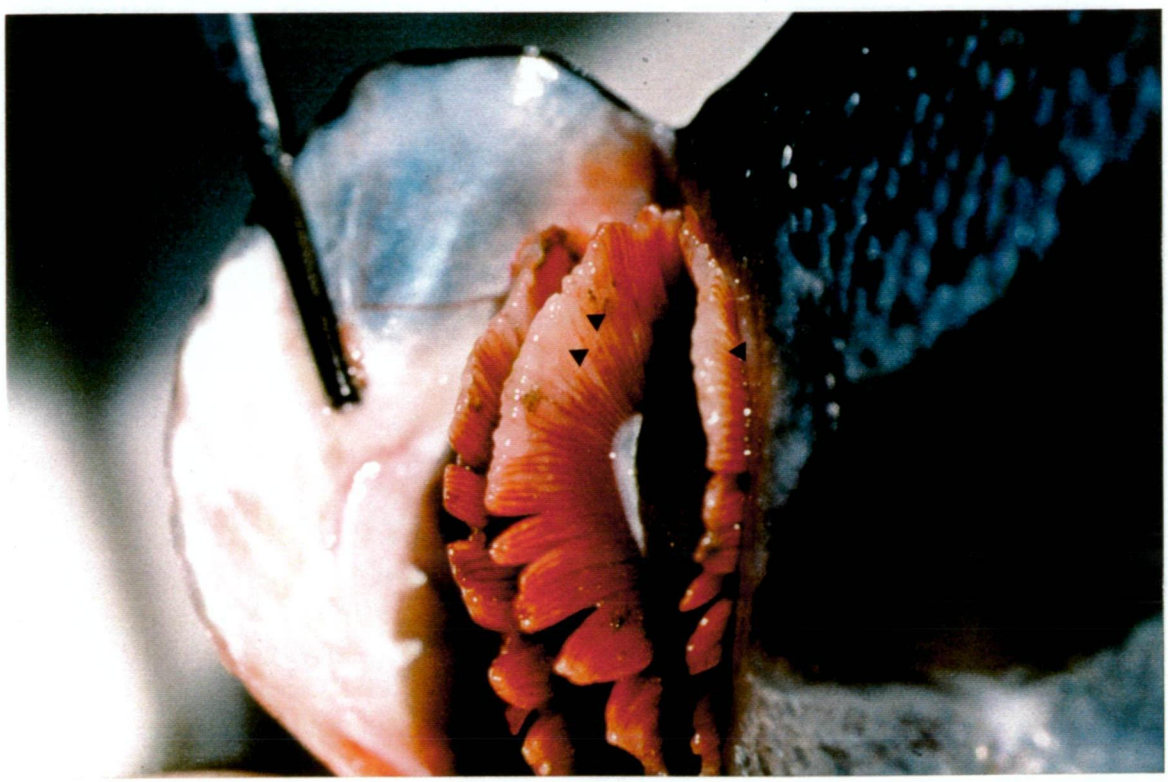


Figure 3.2: Exposed gills of an Atlantic salmon specimen removed from a fishfarm in the *G. catenatum* infected area of the Huon River. Note the exudate-covered and necrotic distal portions of the gill lamellae (arrow head).

Juvenile greenback flounder (*Rhombosolea taparina*) (Fig. 3.3) were used as a standard test fish in exposures with *G. catenatum*. *R. taparina* Gunther 1862, is one of four species of the genus *Rhombosolea* belonging to the subfamily *Rhombosoleinae* of the order Heterosomata. All four species are represented in New Zealand but *R. taparina* is the only member occurring in Australian waters (Kurth 1954). *R. taparina* is confined to the southern states of Australia including Tasmania, and the South Island of New Zealand being common in all these localities. It is the most important commercial flatfish in Victoria and Tasmania with research efforts to establish its suitability for aquaculture. It is for this reason, i.e. ease of availability, that it was

chosen as a test fish. It also responds well to being held captive and is an easy fish to dissect.

The following exposure experiments document the histopathological effect of pure cultures of a Tasmanian strain of *G. catenatum* (GCDE-08) on the greenback flounder *R. taparina*, the results of which have implications for solving the aetiology of the CNG syndrome in Tasmanian sea-caged Atlantic salmon.

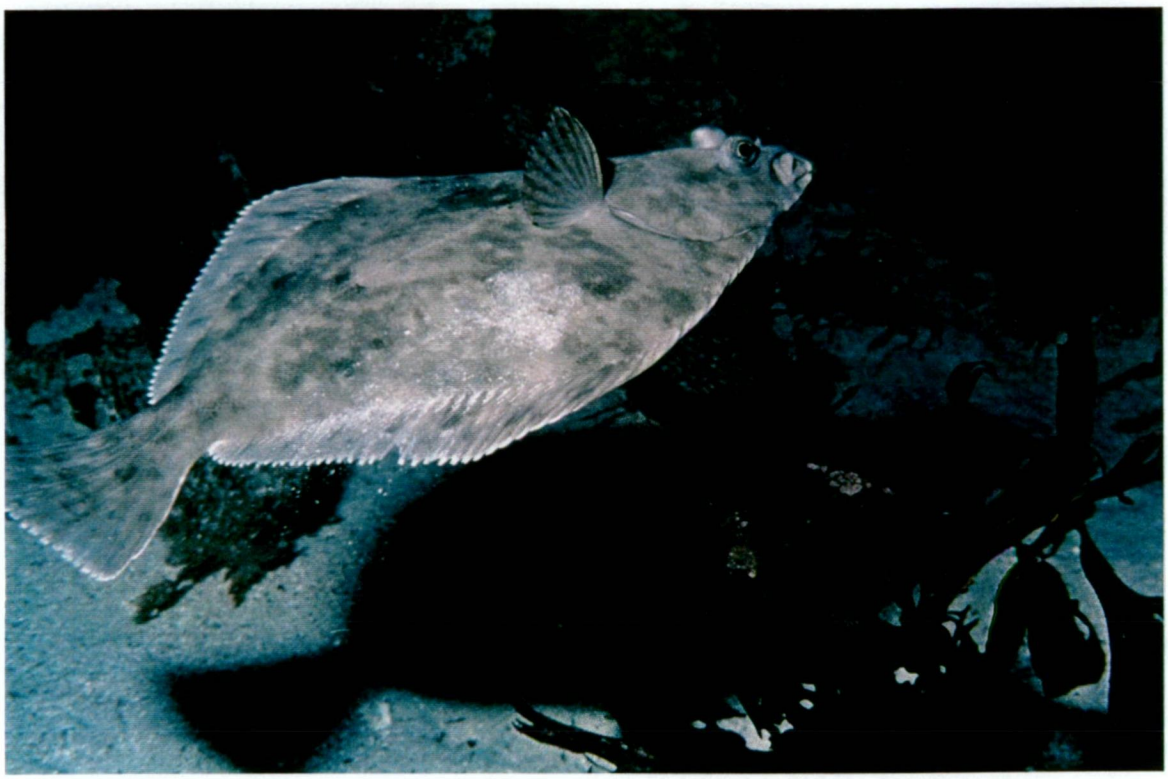


Figure 3.3: Juvenile greenback flounder *Rhombosolea taparina* in its natural habitat.

3.2 Material and Methods

3.2.1 Algal Cultures

Cultures (600-1000mL) of *G. catenatum* (GCDE-08, see Table 2.1) were grown under controlled conditions (12hr light/12hr dark, 17°C) in modified, 30ppt salinity, Gse culture medium (Appendix 1) to late exponential phase and then harvested. Cultures of larger volumes were not grown as *G. catenatum* does not respond well to being grown in large vessels. To obtain cell-free medium the cultures were gently gravity filtered through 5µm plankton gauze and then 0.45µm GF/F glass filters. The filtered culture medium was retained in a clean, sterile

20L glass or polycarbonate carboys at 4°C in the dark. Where solvent extracts were made, filters were retained also for extraction. For live exposures, cultures were used directly.

3.2.2 Lipophilic toxin extraction

Lipophilic toxin extracts were prepared because hemolytic toxin fractions were previously isolated from *G. pulchellum* by Onoue & Nozawa (1989) that caused gill oedema and death in fish. Hence lipophilic extracts were made of *G. catenatum* using a modification of the method of Rhodes (1994). Glass filters (with cells) were soaked in a small amount (100mL) of sterile seawater with the seawater and filters then being frozen for 24h. Once removed from the freezer the frozen mixture was thawed after 100mL of methanol had been added. The filters were removed after being washed twice more with 100ml methanol giving a final methanol volume of 300ml. This mixture of algal cells, seawater and methanol was filtered after 24h through 0.45µm GF/F glass filters into a 1L evaporating bulb. The methanol was removed in a rotovapor at 30°C. The aqueous remainder was then placed in a separating funnel and 100ml of dichloromethane added. The funnel was inverted gently through 180°, 15 times, to gain an adequate mixing of the dichloromethane into the aqueous layer. The bottom dichloromethane layer was then separated off into an evaporating bulb. This separation procedure was repeated thrice giving a final dichloromethane volume of 300mL which was evaporated to dryness in a rotovapor at 30°C. The remaining dry extract was dissolved in a small volume of ethanol (3.6 mL) and stored at 4°C in darkness.

3.2.3 Fish exposures

Flounder, of approximately 30mm length, where obtained from the Key Centre for Aquaculture, Launceston, Tasmania. Individual fish were placed in small aquaria (500mL) with a total of six aquaria per treatment. Aquaria with such small volumes were used as due to the growth constraints of *G. catenatum*, culture material was limited. Exposure aquaria contained either whole cell culture, culture-filtrate or lipophilic solvent extracts (500ml seawater plus 600µL of lipophilic extract) of *G. catenatum*. All exposures lasted for a maximum of 6 days unless fish required euthanasia or died beforehand. Aquaria were illuminated on a 12hr light/12hr dark cycle. Controls consisted of clean seawater containing GSe growth medium. Aquaria were also shielded by a black plastic blind to reduce stress and all aquaria were kept at

a constant 17°C. Daily water changes of 30% were made in all aquaria. A subsample of the removed water, in all cases, was filtered (0.45μm) and assayed for dissolved oxygen, pH and total dissolved ammonia (Hach DR/2000 Spectrophotometer, Salicylate method).

Fish were checked with a torch covered by red cellophane every four hours to observe behaviour, condition and mortality. Any fish dying during the experimental procedure were immediately fixed in 10% seawater formalin. At the end of the experimental period all fish remaining alive were also fixed (10% seawater formalin) after being euthanased with 200ppm benzocaine. After fixation the gills, liver, intestines, kidney and heart of the fish were dissected out infiltrated and then embedded in parafin wax in sectioning blocks. Sections (5μm) were then cut of all tissue with the sections being hand stained (haematoxylin/eosin=H&E, alcian blue =AB-gills only)(Appendix 3) and permanent mounts made.

3.3 Results

3.3.1 Water quality

All total dissolved ammonia concentrations were converted to the proportion of ammonia (NH_3) and ammonium (NH_4^+) using tables calculated by Emerson *et al.* (1975) and later Bower and Bidwell (1978). The figures used were based on a constant salinity of 28-31ppt and a constant temperature of 17°C (Table 3.1). Unionized ammonia levels remained low for the duration of the experimental period with the highest reading being a control at 0.037 mgL^{-1}. The majority of experimental aquaria were below 0.010 mg L^{-1} ammonia. Dissolved oxygen levels remained in a tolerable range of 6.92 to 8.38 mgL^{-1} in most cases with only two fish dying, both in the whole cells group, one in the night of the first day and another on the 5th day due to air pump failures. pH levels remained in a range restricted to approximately 7.0-8.0.

3.3.2 Behaviour and macroscopic examination

Over the six day experimental period only two fish mortalities were recorded (whole cells aquaria 4 on the night of day 1 and whole cells aquaria 2 on the night of day 5). Both fish died due to failure of air pumps. Besides these mortalities all fish appeared calm, resting on the bottom of the aquaria, interspersed with slow swimming periods. Opercular ventilation of the gill cavity was gentle and rhythmic and there were no sudden bursts of agitated movement.

Table 3.1: Average (SD) pH, total dissolved ammonia (TA), ammonia (NH_3)(UI-A) and oxygen concentrations in the experimental and control aquaria over the six day period. Average data are presented for the entire 6 day exposure.

Treatment	Average pH	Average TA (mg/L)	Average UI-A (mg/L)	Average O2 (mg/L)
whole cells 1	8.18 ± 0.05	0.31 ± 0.15	0.0098 ± 0.0046	8.13 ± 0.85
whole cells 2	8.25 ± 0.09	0.08 ± 0.02	0.0025 ± 0.0007	7.37 ± 3.15*
whole cells 3	8.18 ± 0.04	0.10 ± 0.05	0.0035 ± 0.0019	7.82 ± 0.35
whole cells 4	8.20 ± 0.00	0.31 ± 0.00	0.0120 ± 0.0000	4.50 ± 4.81*
whole cells 5	8.22 ± 0.07	0.06 ± 0.05	0.0020 ± 0.0017	8.27 ± 1.24
whole cells 6	8.24 ± 0.05	0.08 ± 0.05	0.0031 ± 0.0018	8.38 ± 0.85
cell-free 1	7.83 ± 0.09	0.38 ± 0.09	0.0055 ± 0.0014	7.28 ± 1.05
cell-free 2	7.83 ± 0.06	0.34 ± 0.14	0.0053 ± 0.0022	7.25 ± 1.24
cell-free 3	7.78 ± 0.11	0.38 ± 0.16	0.0054 ± 0.0030	7.12 ± 1.23
cell-free 4	7.83 ± 0.10	0.22 ± 0.15	0.0038 ± 0.0028	7.12 ± 1.29
cell-free 5	7.79 ± 0.07	0.44 ± 0.13	0.0065 ± 0.0021	7.10 ± 1.06
cell-free 6	7.74 ± 0.10	0.33 ± 0.21	0.0048 ± 0.0018	7.27 ± 0.93
lipophilic 1	7.60 = 0.22	0.10 ± 0.12	0.0012 ± 0.0016	7.00 ± 0.89
lipophilic 2	7.51 ± 0.24	0.41 ± 0.47	0.0048 ± 0.0056	6.93 ± 0.48
lipophilic 3	7.45 ± 0.25	0.49 ± 0.47	0.0053 ± 0.0052	6.95 ± 0.53
lipophilic 4	7.59 ± 0.28	0.11 ± 0.15	0.0013 ± 0.0018	6.92 ± 0.45
lipophilic 5	7.51 ± 0.22	0.23 ± 0.31	0.0025 ± 0.0033	7.23 ± 0.58
lipophilic 6	7.53 ± 0.29	0.33 ± 0.54	0.0016 ± 0.0023	7.02 ± 0.58
control 1	7.75 ± 0.09	0.05 ± 0.05	0.0007 ± 0.0007	7.47 ± 0.81
control 2	7.78 ± 0.12	0.10 ± 0.12	0.0012 ± 0.0012	7.25 ± 0.78
control 3	7.81 ± 0.11	0.25 ± 0.22	0.0037 ± 0.0032	7.33 ± 1.22
control 4	7.84 ± 0.13	0.46 ± 0.44	0.0093 ± 0.0108	7.12 ± 0.91
control 5	7.84 ± 0.09	1.31 ± 0.41	0.0230 ± 0.0107	7.22 ± 1.15
control 6	7.83 ± 0.09	0.42 ± 0.27	0.0072 ± 0.0061	7.08 ± 1.11

Note: * = Fish died due to air pump failures.

In all cases n = 6.

Upon euthanasia at the end of the experimental period, macroscopic examination of the whole animal showed them to be healthy looking. Fins were entire showing no signs of necrosis and the skin had a normal green/grey mottled colour (see Fig. 3.3) also with no signs of necrosis or ectoparasites .External mucous appeared normal and the eyes seemed clear with no discoloration.

3.3.3 Microscopic examination

No pathological lesioning in the fish resembling the CNG syndrome or otherwise was found in any tissue of all treatments. In all cases, gills were entire with no necrosis or clubbing of the gill lamellae nor was there a preponderance of mucus when compared to the controls (Fig. 3.4) in any treatment (Figs 3.5-3.7). Tissues of the liver (Figs 3.8-3.11), intestines (Figs 3.12-3.15), kidney (Figs 3.16-3.19) and heart (Figs 3.20-3.23) were normal with no apparent lesioning or necrosis in any of the treatments when compared to the controls (Figs 3.8, 3.12, 3.16 & 3.20). Some intestine sections showed post-mortem autolysis of the mucosa. This may have been due to the action of enzymes still present in the lumen and a retarded penetration of the fixative (Takashima & Hibiya 1995) as no formaldehyde was introduced directly into the intestines. There was some epithelial lifting in gills but this was thought to be an artifact of fixation due to its common appearance in both treated and control gills alike.

3.4 Discussion

The presumptive link between *G. catenatum* and the CNG syndrome in fish was due to the close temporal coincidence of the disease and blooms of this toxic dinoflagellate. The present work using pure cultures of *G. catenatum* does not support the link. Previous exposure experiments by Lush & Hallegraeff (1993) indicating gill pathology in guppies similar to the CNG syndrome used natural water from the *G. catenatum* infected areas, collected during the massive 1993 bloom. The current experiment indicates that it was not in fact the *G. catenatum* in the water column causing the CNG effect but something else either in association with *G. catenatum* or some unrelated and separate factor, although, again this could be a species effect.

According to Clark *et al.* (1997), the CNG syndrome may well reflect a uniform response to a range of agents or insults. This is in accord with Mallatt (1985) who describes gill changes such as epithelial lifting, epithelial hyperplasia, mucous cell proliferation, lamellar fusion and telangiectasia, that are reported frequently in the literature (Daoust *et al.* 1984, Gehrke *et al.* 1993, Bullock *et al.* 1994, Ingersoll *et al.* 1990), to be stereotypical and limited pathological responses of fish gills to stresses such as water-borne or blood-borne toxins. *G. catenatum*'s role in the CNG syndrome is not supported by the laboratory experiment with flounder. Similarly, bioassays using filtered *G. catenatum* culture medium (Chapter 2) were non-toxic to 4-day old brine shrimp, *Artemia salina* or the non-toxic phytoplankton *Tetraselmis suecica.*

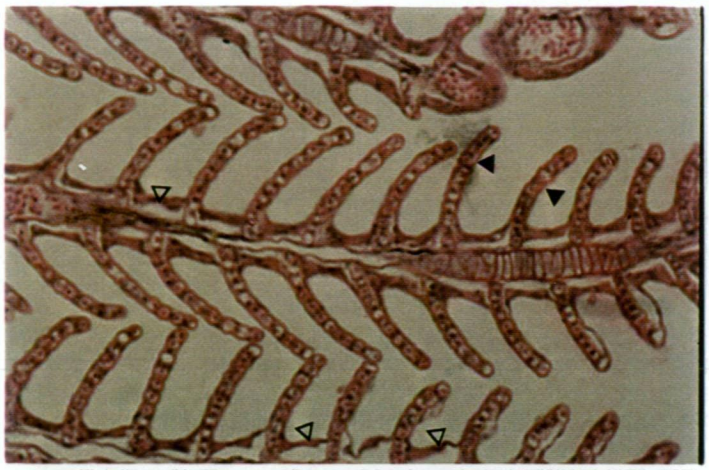


Figure 3.4: Gills of *R. taparina* exposed to clean seawater (+GSe) (control). H&E stain x 320, Δ: lifting of the epithelium and the interlaminar cells, ▲: normal thickness respiratory epithelium and the absence of lamellar fusion.

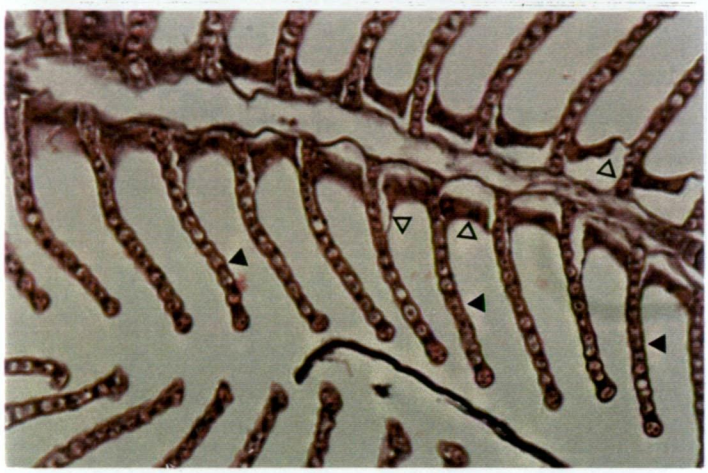


Figure 3.5: Gills of *R. taparina* exposed to cell-free, *G. catenatum* culture medium. H&E stain x 400, Δ: lifting of the epithelium and the interlaminar cells, ▲: normal thickness respiratory epithelium and the absence of lamellar fusion.

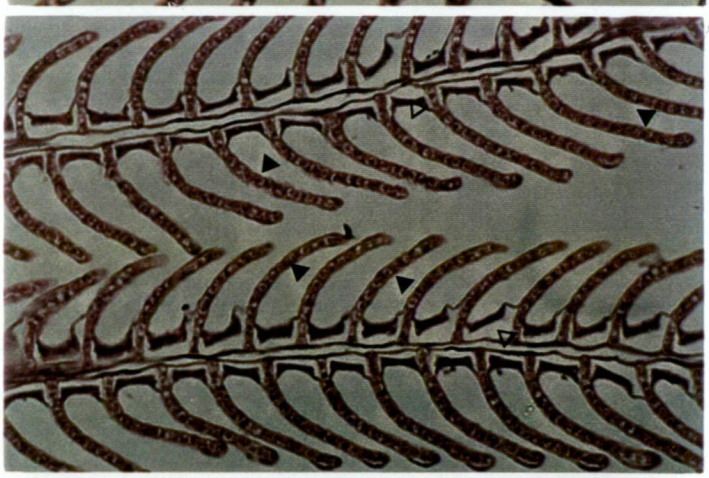


Figure 3.6: Gills of *R. taparina* exposed to whole cell, *G. catenatum* culture. H&E stain x 250, Δ: lifting of the epithelium and the interlaminar cells, ▲: normal thickness respiratory epithelium and the absence of lamellar fusion.

Figure 3.7: Gills of *R. taparina* exposed to lipophilic extract of *G. catenatum* culture dissolved in seawater. H&E stain x 320, Δ: lifting of the epithelium and the interlaminar cells, ▲: normal thickness respiratory epithelium and the absence of lamellar fusion.

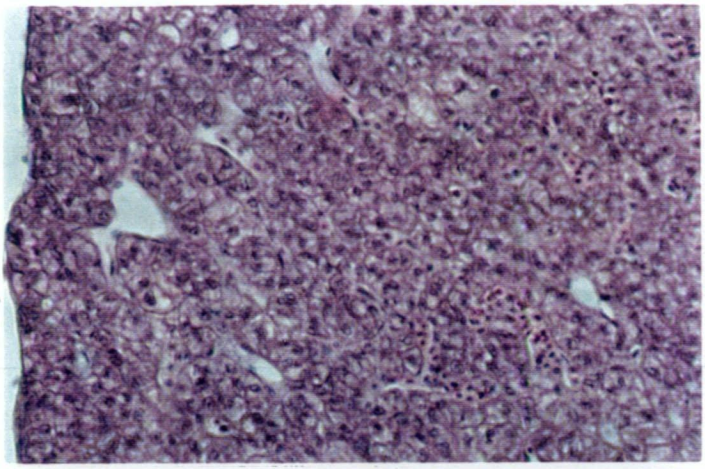


Figure 3.8: Liver from *R. taparina* exposed to clean seawater with the addition of GSe nutrients (control). H&E stain x 625.

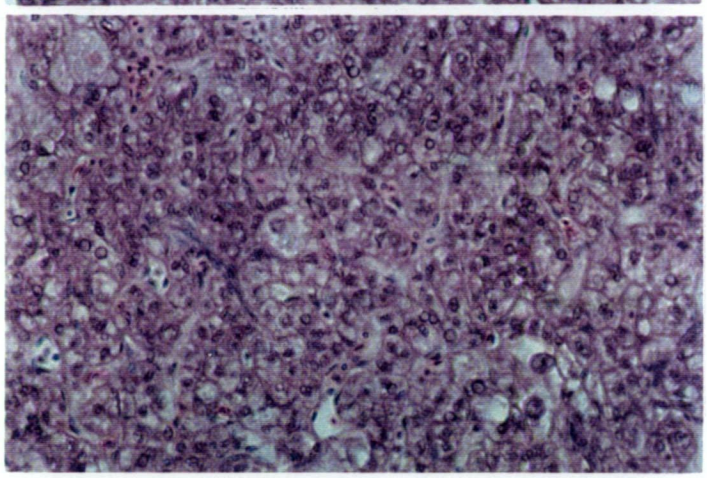


Figure 3.9: Liver from *R. taparina* exposed to cell-free, *G. catenatum* culture medium. H&E stain x 625.

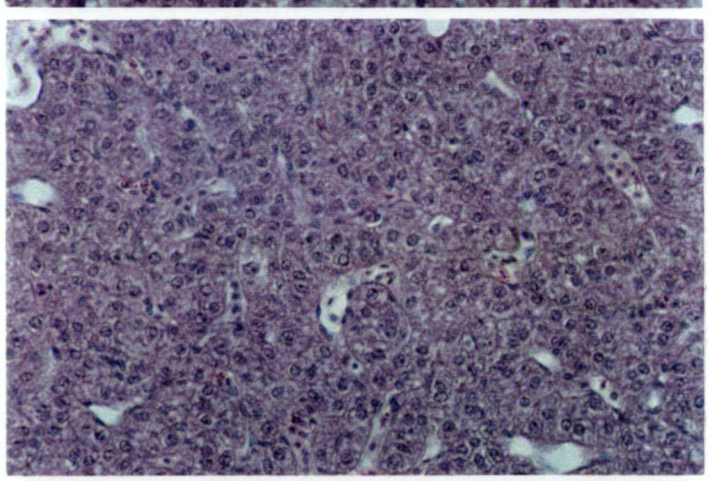


Figure 3.10: Liver from *R. taparina* exposed to whole cell, *G. catenatum* culture. H&E stain x 625.

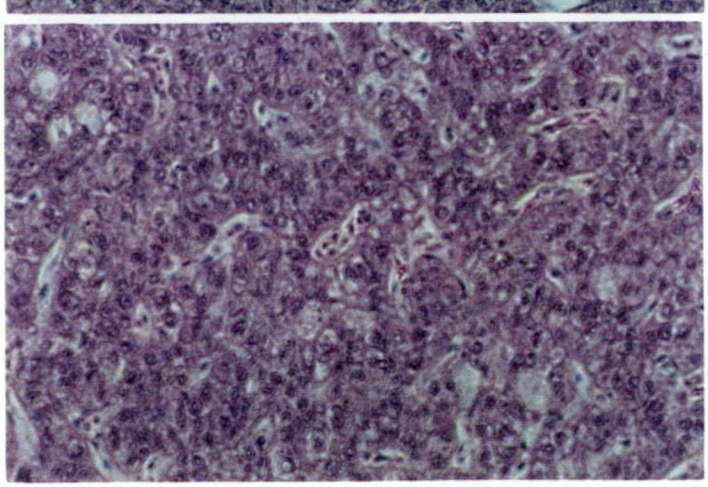


Figure 3.11: Liver from *R. taparina* exposed to lipophilic extract of *G. catenatum* culture dissolved in seawater. H&E stain x 625.

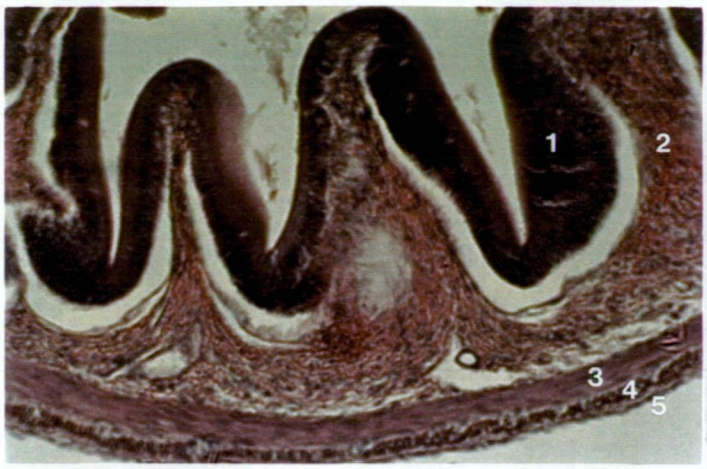


Figure 3.12: Anterior intestinal tissue from *R. taparina* exposed to clean seawater with the addition of GSe nutrients (control). H&E stain x 250, 1: mucosal epithelium, 2: submucosa (stratum compactum & stratum granulosum), 3: circular muscle layer, 4: longitudinal muscle layer, 5: outer membrane (tella subserosa & serous membrane).

Figure 3.13: Anterior intestinal tissue from *R. taparina* exposed to cell-free, *G. catenatum* culture medium. H&E stain x 250, 1: mucosal epithelium, 2: submucosa (stratum compactum & stratum granulosum), 3: circular muscle layer, 4: longitudinal muscle layer, 5: outer membrane (tella subserosa & serous membrane).

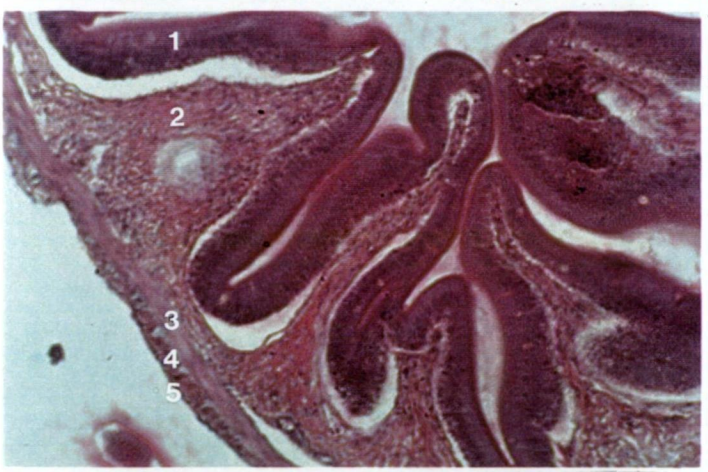


Figure 3.14: Anterior intestinal tissue from *R. taparina* exposed to whole cell, *G. catenatum* culture. H&E stain x 250, 1: mucosal epithelium, 2: submucosa (stratum compactum & stratum granulosum), 3: circular muscle layer, 4: longitudinal muscle layer, 5: outer membrane (tella subserosa & serous membrane).

Figure 3.15: Anterior intestinal tissue from *R. taparina* exposed to lipophilic extract of *G. catenatum* culture. H&E stain x 158, 1: mucosal epithelium, 2: submucosa (stratum compactum & stratum granulosum), 3: circular muscle layer, 4: longitudinal muscle layer, 5: outer membrane (tella subserosa & serous membrane).

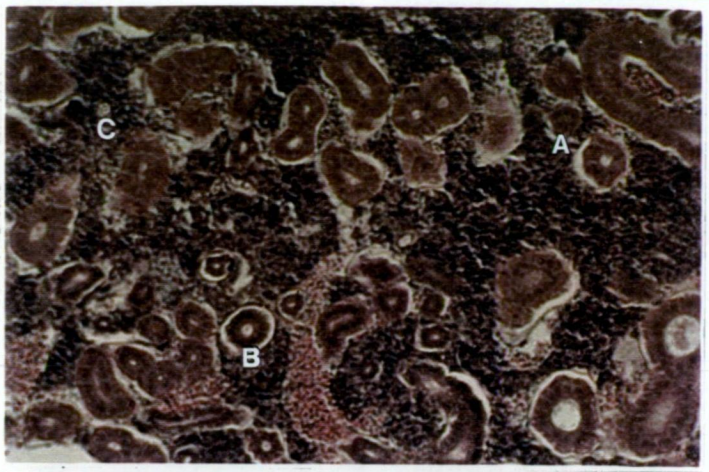


Figure 3.16: Kidney tissue from *R. taparina* exposed to clean seawater with the addition of GSe nutrients (control). H&E stain x 250, A: glomerulus, B: renal tubule, C. lymphoid tissue.

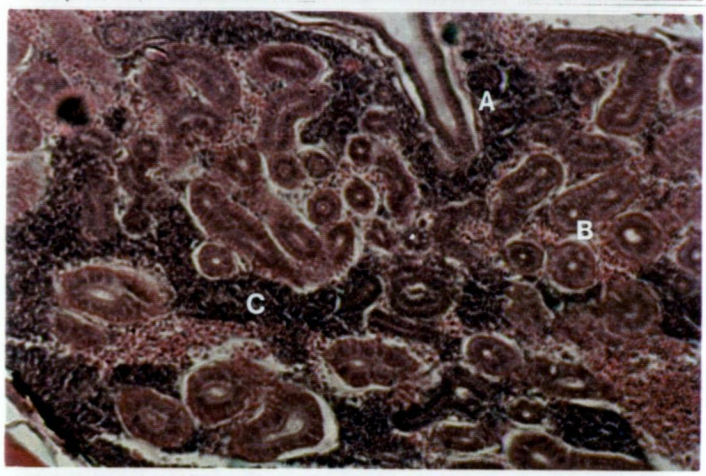


Figure 3.17: Kidney tissue from *R. taparina* exposed to cell-free, *G. catenatum* culture medium. H&E stain x 250, A: glomerulus, B: renal tubule, C. lymphoid tissue.

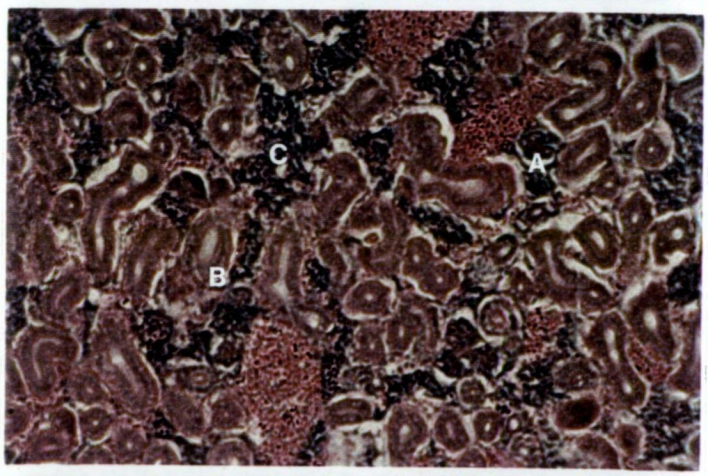


Figure 3.18: Kidney tissue from *R. taparina* exposed to whole cell, *G. catenatum* culture. H&E stain x 250, A: glomerulus, B: renal tubule, C. lymphoid tissue.

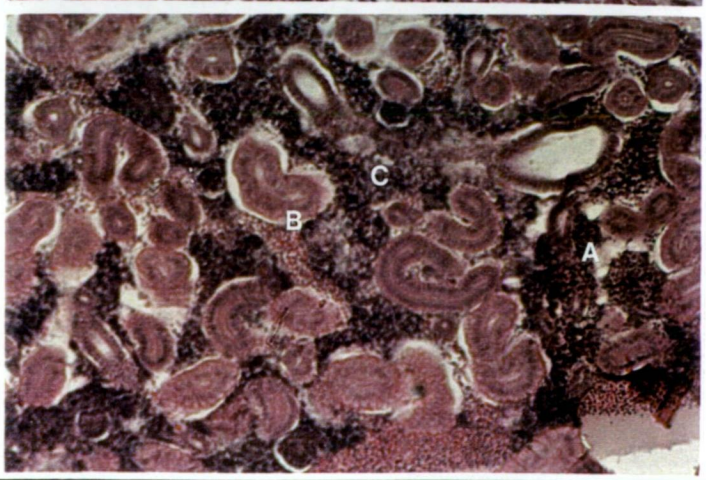


Figure 3.19: Kidney tissue from *R. taparina* exposed to lipophilic extract of *G. catenatum* culture. H&E stain x 250, A: glomerulus, B: renal tubule, C. lymphoid tissue.

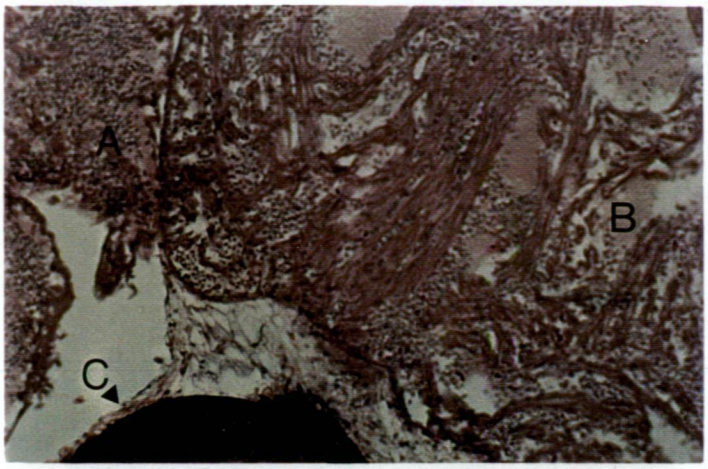


Figure 3.20: Heart tissue from *R. taparina* exposed to clean seawater with the addition of GSe nutrients (control). H&E stain x 250, A: atrium, B: ventricle, C. bulbus arteriosus

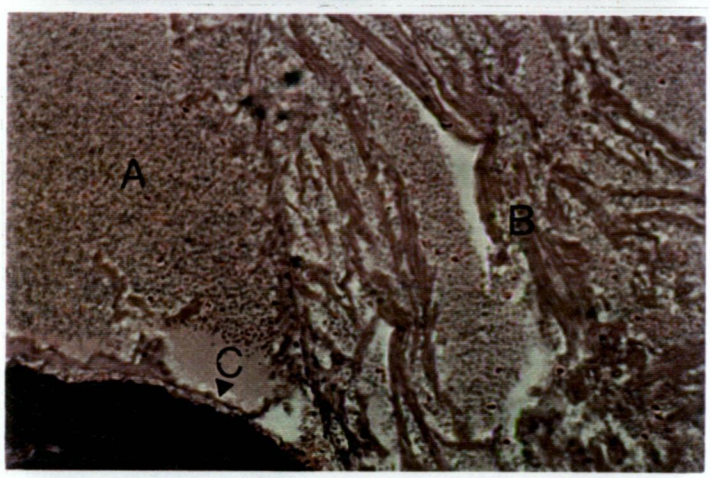


Figure 3.21: Heart tissue from *R. taparina* exposed to cell-free, *G. catenatum* culture medium. H&E stain x 250, A: atrium, B: ventricle, C. bulbus arteriosus

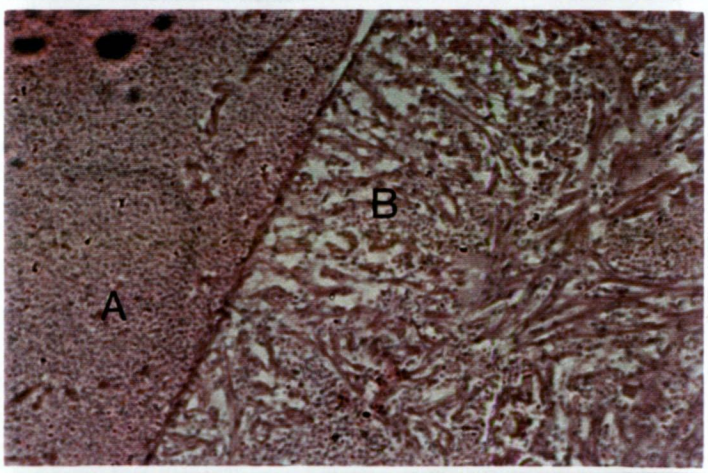


Figure 3.22: Heart tissue from *R. taparina* exposed to whole cell, *G. catenatum* culture. H&E stain x 250, A: atrium, B: ventricle.

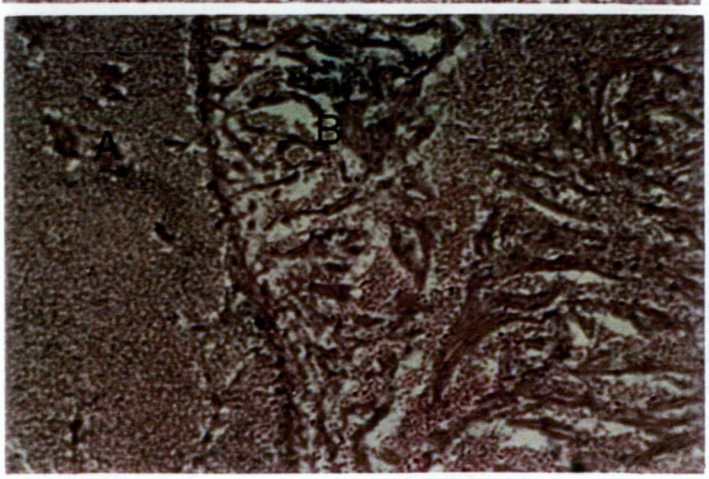


Figure 3.23: Heart tissue from *R. taparina* exposed to lipophilic extract of *G. catenatum* culture. H&E stain x 250, A: atrium, B: ventricle.

Other factors have been proposed to account for the CNG syndrome, but none have yet been proven. Some researchers (B. Reguera pers. comm.) have indicated that *G. catenatum* is a producer of non-toxic mucilages that could effect fish gill respiratory potential via changes in culture viscosity (Jenkinson 1986), however this did not appear to be the case here as no gill changes or mortality was recorded. The fact that fish with the CNG disease recover without any specific treatment (Munday, 1995 personal communication) and the absence of pathogenic organisms (viral, bacterial and parasitic) in histological sections or smears (Clark *et al.* 1997) suggests gill changes induced by environmental factors, particularly as during the 1993 outbreak the disease seemed to spread with the currents seaward. Heavy metals (Daoust *et al.* 1984, Mueller *et al.* 1991), insecticides (Dutta *et al.* 1993) and herbicides (Morgan & Kiceniuk 1992) have all been reported to produce gill pathology similar to that found in the CNG syndrome. Ammonia, in higher concentrations and particularly combined with low pH (Sarjito 1993), also causes gill pathology similar to the CNG syndrome, however, as yet, detailed chemical analysis of the water column during outbreaks of the CNG syndrome is lacking.

A dietary pantothenic acid (PA) deficiency (nutritional gill disease) has been proposed as an alternative for the CNG syndrome since a lack of this co-enzyme can lead to marked lamellar hyperplasia (Karges & Woodward 1984) and fusion of the distal portion of gill secondary lamellae, similar to the CNG syndrome (Lovell 1975, Clark *et al.* 1997). However, the epidemiology of the outbreak does not support a nutritional aetiology. The outbreak was sudden and restricted geographically, while the fish feed pellets used throughout the industry were all of the same origin (B. Munday: pers. comm.). Also, the affected fish recovered from the disease without dietary modification and feeding trials with affected fish using feed fortified with PA and vitamin C had no affect on the condition compared to untreated fish (S. Percival: pers. comm.).

Even though the CNG syndrome aetiology is still unknown, several factors may have had a predisposing or modifying effect on the CNG condition. Intrinsic factors such as the physiological state and overall health of the fish, water quality variables (dissolved oxygen concentration, water flow, temperature) and biotic factors (coelenterates, i.e jelly fish and hydroids with stinging nematocysts, other algal blooms) could all induce a state of stress, rendering the fish more susceptible to disease from other sources.

Chapter 4: Ichthyotoxicity and cytotoxicity of *A. minutum* whole cells and filtered culture medium

4.1 General Introduction

A variety of micro algal species belonging to the Prymnesiophyceae (*Chrysochromulina, Prymnesium*), Raphidophyceae (*Chattonella, Heterosigma*), Dinophyceae (*Gymnodinium breve, G.* cf. *mikimotoi*) and Cyanophyceae (*Anabaena circinalis*) are known to release substances into the surrounding seawater (Larsson & Hagstrom 1982, Amouroux 1984, Gentien 1998) that can represent 70% of the primary production lost by cells (Chrost & Faust, 1983). Some of these exocellular substances have been found to be toxic (Ogata & Kodama, 1986, Hallegraeff *et al.*1995). Such exotoxins may have an allelopathic role in the chemical warfare against competing phytoplankton, such as that proposed for *G. mikimotoi* (reported as*G.* cf. *nagasakiense*, Gentien & Arzul 1990, and *Gyrodinium aureolum*, Yasumoto *et al.* 1990, Parrish *et al.* 1993), as well as function as an antiherbivory defence against zooplankton and planktivorous larval fish. However, ichthyotoxic exudates have not been widely reported in the PSP dinoflagellate genera *Alexandrium*, where food chain effects are more commonly the result of transvectorial transfer of PSP toxins from filter or planktivorous feeding organisms (zooplankton, larval fish) to higher trophic carnivores (White 1980; White 1981). Exceptions to this are the claimed involvement of *A. tamarense* (=*Gonyaulax excavata*) in a fish kill of farmed salmon and rainbow trout in the Faroe Islands (Mortensen 1985), the demonstration of inimical effects of culture exudates on the behaviour and growth of tintinnids (Hansen 1989) and lethal effects of *A. tamarense* to surf smelt *Hypomesus pretiosus japonicus* (Ogata & Kodama 1986). Recently we demonstrated the potent, toxic-exudate effects of cell-free *A. minutum* growth medium on the brine shrimp *Artemia salina* (Lush & Hallegraeff 1996), while Bagoien *et al.* (1996) demonstrated similar effects of *A. minutum* on the harpacticoid copepod *Euterpina acutifrons*. This laboratory evidence supports claims by Su *et al.* (1993) that a Taiwanese strain of *A. minutum* (reported as *A. tamarense*) was linked to prawn mortality in coastal ponds. In Egyptian coastal waters, mortality of wild fish and of fish kept in aquariums with filtered seawater has also been linked to blooms of *A. minutum* (Halim & Labib 1996). There also exists anecdotal evidence for the association between pilchard kills in the Port River, South Australia, and *A. minutum* blooms in the same area (K. Jones pers. com.).

Ichthyotoxicity is a rather loose term used to generally indicate the lethal toxicity of an alga towards aquatic organisms such as fish. The gills are one of the sites through which waterborne toxicants enter the bodies of fish, and the gills are often among the organs most affected (Skidmore & Tovell 1972; Khangarot 1982; Chevalier *et al.* 1985) due to their direct contact with the seawater and their highly delicate nature (Eller 1975). It is often extremely difficult to establish which toxins are present as frequently recorded histopathological lesions and changes in gill epithelia (lifting, necrosis, hyperplasia, hypertrophy, rupture), bulbing or fusing of gill lamellae, hypersecretion and proliferation of mucocytes, and changes in chloride cells and gill vasculature are largely nonspecific and occur under many different exposure conditions (Mallatt, 1985). However, they do indicate that a toxicant or irritant is present and many of the responses are primarily stereotypical patho-physiological responses that could be considered defensive (Mallatt 1985).

Species of the genus *Alexandrium* are well established as major producers of PSP toxins, particularly the highly potent carbamate toxins Gonyautoxin 1-4 (GTX_1-GTX_4) (see Chapter 1). PSP toxins exert their toxic effects by binding to a site on the exocellular surface of the voltage activated sodium channel in neurons blocking (by unknown means but hypothesised to be physical obstruction) the passive inward flux of sodium ions occurring during conductance (Hall *et al.* 1990); they do not damage the neuron *per se*. Hence, PSP toxins are considered neurotoxic but not cytotoxic, and PSP toxins are not known to produce pathological lesions in marine fauna (Oshima, pers. comm.). However, three members of the genus *Alexandrium* (*A. tamarense, A. catanella, A. monilata*) have also been observed producing powerful hemolytic effects (Gates & Wilson 1960, Shilo 1971, Erker *et al.* 1985, Underdal *et al.* 1989, Simonsen *et al.* 1995) when extracts of them have been presented to erythrocytes of different animals. Gates & Wilson (1960) also found the filtered culture media of *A. monilata* to be toxic to fish while Erker *et al.* (1985) observed *G. monilata* causing necrosis of longitudianal muscle tissue in the intestines of fish and causing fish kills in the Gulf of Maine. Hemolysins are cytolytic toxins, so named for their ability to lyse erythrocytes via enzymatic disruptions of target cell membranes, insertion into target membranes forming pores leading to cellular lysis and solubilization of cell membranes by formation of surfactants (Rowe & Welch 1994). The vast majority of hemolytic cytotoxins are toxic to other cell types as well and, indeed, their action against erythrocytes may be largely coincidental.

The following chapter reports on experiments conducted to investigate the apparent ichthyotoxic nature of *A. minutum* whole-cell culture and culture-filtrate to the estuarine flounder *Rhombosolea taparina.* Effects of *A. minutum* are observed in *R. taparina* in terms of pathological damage to major tissue structures, particularly the gills, physiological changes in gill respiratory enzyme activity (SDH) and blood chemistry. These experiments are presented in two distinct sections. Section 4.3 deals purely with the histopathological effects observed in *R. taparina* on exposure to *A. minutum* and pure GTX toxins. Section 4.4 reports on clinical changes in the blood (blood PCV, concentration of blood plasma ions and proteins [sodium, chloride, potassium, albumin and globulins] and blood plasma osmolality) and the gill chloride cell enzyme, succinic dehydrogenase (SDH) of challenged fish. Materials and Methods common to both experiments are presented first as is a General Introduction. Both sections contain explanations of protocols unique to them and also dedicated Results and Discussion sections. All results are discussed at the end in a General Discussion section in terms of the possible nature of the toxic principle involved and it's impacts via natural blooms of this algae on wild and cultured fish stocks.

4.2 General Material and Methods

4.2.1 Algal cultures

Batch cultures (20L) of *A. minutum* were grown under controlled conditions (12hr light/12hr dark, 17°C) in modified, 30ppt salinity, Gse culture medium (Appendix 1) to mid exponential phase and then harvested. Cultures were only harvested when cell densities reached approximately 10^5 ml^{-1}, which corresponded to the mid-exponential phase, and harvesting always took place at midday. To obtain culture-filtrate the cultures were gently gravity filtered through 5µm plankton gauze and then 0.45µm GFF glass filters. The filtered culture medium was retained in clean, sterile 20L glass or polycarbonate carboys at 4°C in the dark. Where solvent extracts were made, filters were retained with cell remains. For live exposures, cultures were used directly.

4.2.2 Fish exposures

Live juvenile greenback flounder, of approximately 30mm (histology only) and 100mm length

(histology and clinical pathology), where obtained from the Department of Sea Fisheries, Taroona Laboratories, Crayfish Point, Taroona, Tasmania. All fish were held in a large 200L aquaria filled with clean seawater, at 17°C, for at least 24 hours before being introduced into the experimental conditions (Table 4.1). Fish of 30mm length were exposed in 10L aquaria (except for fish exposed to GTX toxins which were exposed in specially constructed 1L aquaria), and fish of 100mm length were exposed in 45L aquaria. Fish were not fed just prior nor during the experimental periods to reduce ammonia buildup and all exposure aquaria were shielded with black plastic blinds. Visual checks of fish behaviour and mortality were conducted at regular intervals using a torch.

Exposure aquaria contained either whole cell culture, culture-filtrate or lipophilic solvent extracts of *A. minutum* (Table 4.1). Fish (100mm) were also exposed to 15%, 30% and 60% strength, cell-free *A. minutum* medium (mixed with clean, sterile seawater with the addition of GSe nutrients) and pure $GTX_{(1-4)}$ toxins (30mm fish only)

Monitoring of water quality, environmental conditions, euthanasia, dissection and tissue preparation was per Chapter 3 (section 3.2.3) and Appendix 3. Fish were exposed for varying times and to varying treatments of *A. minutum* (Table 4.1). All control treatments consisted of clean, 0.2µm filtered (Gelman, 90mm supor-200 membranes) seawater with the addition of GSe growth nutrients (Appendix 1).

Table 4.1: Summary of fish exposure conditions, number and size of fish and time exposed.

Trial	Treatment	No. of fish exposed	No. of control fish	Exposure time (hrs)	Fish length (mm)
A	whole-cells	12	6 *	24	30
	cell-free medium	12	6 *	24	30
	lipophilic extract	6	6 *	120	30
B	cell-free medium	6	6	2.5	≥100
C	cell-free, 60% strength	9	3 **	10	≥100
	cell-free, 30% strength	10	3 **	10	≥100
D	cell-free, 15% strength	6	3	144	≥100
E	pure GTX(1-4) toxins	6	2	24	30

Note: * and ** = denote the same controls for one exposure experiment.

4.3: Microscopic gill lesions induced in fish by *A. minutum* whole cells and filtered culture medium[2].

4.3.1 Introduction

Pathological changes in fish tissues are commonly the result of exposure to toxins or toxicants. Although these tissue changes are present they are more often than not broad-ranging stereotypical responses rather than specific effects bought about by one particular substance (Mallat 1985). Hence, although histopathological lesions in fish exposed to suspect seawater may not resolve that one specific toxin or toxicant is present it does indicate the presence of a toxic substances in the seawater. This was the basis of the following investigation. Fish bioassays were used to assess the toxicity of the growth medium of *A. minutum* and depending on lesion type and location it was hoped that preliminary elucidation of the toxic principle(s) would be determined.

4.3.2 Materials and Methods

Respiratory epithelial distance was measured in fish from Trial A using a microscope-eyepiece graticule. The thickness of four secondary lamellae was measured on nine primary lamellae of two gill arches (2nd & 3rd), giving a total of 36 measurements per gill arch and 72 measurements per fish on all fish exposed in Trial A. Similarly, the numbers of mucocytes were measured on gills in Trial B after the gills had been stained with alcian blue (Appendix 3). The number of mucocytes (stained dark blue) were counted on 10 measured lengths (mm) of secondary lamellae in all fish in Trial B and the number of mucocytes per mm^2 calculated.

4.3.2.1 Pure gonyautoxin (GTX) exposure

Three, 1L tanks were constructed (220 x 110 x 90mm) from clear, 3mm glass and each filled with 1L of clean, sterile seawater (Gelman 0.2µm Supor-200 P/N 60334) containing pure GTX(1-4) toxins (GTX_4: Sigma G-0405, lot no. 27H0634; $GTX_{(1-4)}$: NRC-CNRC, PSP-1B

[2]An earlier version was published as: G. J. Lush, G. M. Hallegraeff and B. L. Munday (1998). Histopathological effects in juvenile greenback flounder *Rhombosolea taparina* exposed to the toxic dinoflagellate *Alexandrium minutum*. In: B. Reguera, J. Blanco, M. L. Fernandez and T. Wyatt (Eds), *Harmful Microalgae*, Xunta de Galicia and IOC of UNESCO, 609-610.

Kits, certified reference materials) at a concentration equalling the molar percentage (i.e. GTX_1 = 45.2%, GTX_2 = 15.7%, GTX_3 = 10.8%, GTX_4 = 27.5%; Hallegraeff *et al.* 1991) of these toxins endocellularly in *A. minutum* but to a maximum toxicity (toxicity in mouse units STX = 2045, GTX_1 = 1638, GTX_2 = 793, GTX_3 = 2234, GTX_4 = 673) equivalent to 97nM STX (Chapter 2, section 2.3.3).

Example calculation for GTX_1:

(GTX_1)(μL) in 97nM STX equiv:

$= 97_{\{max.\ tox.\}} \times (45.2/100)_{\{mole\ \%\ GTX_1\}} \times (2045/1638)_{\{rel.\ tox.\ GTX_1\ to\ STX\}}$
$= 54.74nM$

$= 0.05474\mu M$

$=0.05474\mu M \times 410_{\{mol.\ weight\ GTX_1\}}$

$= 22.44\mu gL^{-1}$ (similarly: $GTX_2 = 15.47\mu gL^{-1}$, $GTX_3 = 3.79\mu gL^{-1}$, $GTX_4 = 33.24\mu gL^{-1}$)

Two fish each were placed in the 1.0L aquaria and exposed for 24h after which they were euthanased, fixed and dissected. Tissue was sectioned and permanent slide mounts made. The control consisted of fish exposed to clean, sterile seawater (Gelman 0.2μm Supor-200 P/N 60334) with the addition of GSe nutrients. An *Artemia* bioassay was also conducted on the seawater after the addition of the $GTX_{(1-4)}$ toxins. The control consisted of clean seawater with the addition of GSe nutrients.

4.3.3 Results

4.3.3.1 Water quality

All total disolved ammonia concentrations were converted to their proportion of ammonia (NH_3) and ammonium (NH_4^+) using tables calculated by Emerson *et al.* (1975) and later

Bower and Bidwell (1978). The figures used were based on a constant salinity of 28-31ppt and temperature of 17°C (Appendix 4).

pH and oxygen concentrations remained relatively stable over the experimental periods with only minor shifts over time (Tables 4.2 & 4.3). pH remained between 7.5 and 8.5 and oxygen between 7.5 and 10.5 mg/ L^{-1} with no clear fluctuations in any one group. Dissolved ammonia can have dramatic effects on the histology of fish gills and can cause rapid mortality. Ammonia (NH_3) and ammonium (NH_4^+) exist in equilibrium dependant upon pH and temperature. This is important as in normal systems it is ammonia that is of particular concern as it is freely diffusible across biological membranes whereas the ammonium is not (Tomasso 1994). Tomasso also indicated that the median lethal dose (LC50) of ammonia to channel catfish (*Ictalurus punctatus* and *Ictalurus* sp.) was 2.4 and 2.6mg L^{-1} with exposure times of 24h and 96h respectively. While Weirich & Tomasso (1993) found the LC50's (96hr exposure) for sunshine bass (female *Morone chrysops* x male *M. saxatilis*) range from 0.32 to 0.60 mg L^{-1}. Concentrations of ammonia in the present study remain well below these levels with the highest recorded concentrations being in the 30% and 60% cell-free media treatments (0.017mg L^{-1}), pure $GTX_{(1-4)}$ treatments (0.025mg L^{-1}) and their respective controls (0.033 and 0.021mgL^{-1}).

Although histological gill changes in response to ammonia exposure have been indicated by other researchers (Larmoyeux & Piper 1973, Smith & Piper 1975, Smart 1976) it is also apparent from the literature that this may be highly species specific in terms of dose and time exposed. Smith & Piper (1975) found pathological changes (hyperplasia and swelling) in the gills and liver of trout, *Oncorhynchus mykiss*, exposed to 1.6mg L^{-1} of ammonia (3.0µg L^{-1}) with exposure times ranging from a few weeks to a few months. Similarly, lamellar hypertrophy was observed in the gills of juvenile *O. mykiss* constantly exposed for 14 days to ammonia at or above 0.03mg L^{-1} and intermittent levels of 0.05mg L^{-1} (Klontz *et al.* 1985). While Soderberg *et al.* (1984) and Robinette (1976) found gill epithelial hyperplasia, and epithelial lifting in the gills of channel catfish, *I. punctatus*, exposed to average daily ammonia levels of 20-67µg L^{-1} (daily maxima 63-183µg L^{-1}) and 120µg L^{-1} (29 day exposure), respectively. In contrast to this, no pathological lesions were found in the gills of rainbow trout, *O. mykiss*, when exposed to 0.2-0.4mg L^{-1} ammonia for 90 days (Daoust & Ferguson 1984). Similarly, turbot, *Scophthalmus maximus* L. and Dover sole, *Solea solea* L., showed no gill damage after exposure of up to 0.6mg L^{-1} ammonia for 42 days (Alderon 1979).

Table 4.2: Average (SD) water quality of experimental and control aquaria in experiment Trials A, B, C and E under 24 hours duration. Averages are for each group over the exposure period.

Trial	Treatment (*A. minutum*)	*n*	Exposure time (hrs)	Average pH	Average TA¶ (mg/L)	Average UIA† (mg/L) (x0.001))	Average O2 (mg/L)
A	Whole-cells	12	24	8.26 ± 0.17	0.03 ± 0.02	1.7 ± 1.1	10.47 ± 0.34
	Cell-free	12	24	8.02 ± 0.04	0.16 ± 0.24	3.9 ± 6.0	7.04 ± 0.53
	Lipophilic	6	24	7.99 ± 0.06	0.27 ± 0.04	6.3 ± 1.2	7.47 ± 0.23
	Control	6	24	7.98 ± 0.05	0.28 ± 0.12	6.5 ± 2.3	7.2± 0.19
B	Cell-free	6	2.5	8.63 ± 0.03	0.13 ± 0.04	9.6 ± 2.8	9.42 ± 0.7
	Control	6	2.5	7.62 ± 0.15	0.32 ± 0.22	3.9 ± 3.4	9.33 ± 0.62
C	30% cell-free	10	10	7.92 ± 0.09	0.63 ± 0.19	10.0 ± 10.0	8.88 ± 0.50
	60% cell-free	9	10	8.10 ± 0.18	0.56 ± 0.18	17.0 ± 1.5	9.27 ± 0.31
	Control	3	10	8.21	0.85	33.3	9.7
E	GTX (1-4)	4	24	7.81 ± 0.04	1.49 ± 0.16	25.8 ± 3.0	10.63 ± 0.97
	Control	2	24	7.9	1.05	21.1	9.9

NB: ¶ = TA or total ammonia (ammonia and ammonium)

† = UIA or ammonia (NH_3)

Table 4.3: Average (SD) water quality of experimental and control aquaria in experimental Trial D using 15% *A. minutum* culture-filtrate to expose fish for 144 hours (6 days). Averages are for the entire exposure period ($n = 6$).

Tank	Average pH	Average O2 (mg/L)	Average TA¶ (mg/L)	Average UIA† (mg/L)
1	7.88 ± 0.10	9.77 ± 0.80	1.02 ±0.13	0.02 ± 0.01
2	7.83 ± 0.13	9.73 ± 0.73	1.05 ± 0.28	0.02 ± 0.01
3	7.85 ± 0.09	9.83 ± 0.84	0.97 ± 0.44	0.02 ± 0.01
4	7.86 ± 0.09	9.67 ± 0.95	0.78 ± 0.18	0.02 ± 0.01
5	7.78 ± 0.11	9.25 ± 0.84	1.35 ± 0.40	0.03 ± 0.01
6 *	7.85 ± 0.12	9.57 ± 1.00	0.97 ± 0.33	0.02 ± 0.01

NB: * = Control aquaria.

¶ = ¶ = TA or total ammonia (ammonia and ammonium)

† = UIA or ammonia (NH_3)

The levels of ammonia in the present study are typically low (0.03mg L^{-1} maximum reading) and even for maximal readings the time of exposure is also typically short (24h or less). The highest readings were found in treatments that did not show any pathological changes to gill or

other tissues (10hr, 30% and 60% control, 24 hr pure $GTX_{(1-4)}$ treatment and control), hence the ammonia levels encountered within this study were considered to be within the normal, tolerable range for this species of flounder at these exposure times.

4.3.3.2 Tissue pathology

4.3.3.2.1 *Macroscopic examination*

High mortalities were observed in fish exposed to whole cell cultures (50% mortality) and culture-filtrate (25% mortality) in Trial A while no mortality was recorded from the lipophilic extracts and only one mortality in the controls (hypoxia from a damaged air pump) (Table 4.4). There was no mortality recorded in fish exposed to 100% culture-filtrate in Trial B or its seawater (+GSe) control however, the exposure time was very short at 2.5h. Mortality in Trial C was also high in fish exposed to 60% culture-filtrate (33% mortality) and 30% culture-filtrate (45%) while there was little or no mortality recorded in the fish exposed to 15% culture-filtrate or those exposed to pure GTX_1-GTX_4 toxins or their respective controls (Table 4.4). Where mortality occurred, it was rapid with no definite sign of its onset making diagnosis and recovery of gill tissue suitable for analysis extremely difficult. Gills degenerated rapidly upon death with the passage of even a few minutes making histological analysis impossible. There was also great variation in individual response to the whole cells culture and cell-free *A. minutum* medium compounding the difficulty of assessing morbidity and permitting recovery of intact tissue.

Treated fish that were found alive were in various states of stress showing signs similar to those reported by Roberts *et al.* (1983) for rainbow trout (*O. mykiss*) exposed to *G.* cf. *mikimotoi* (reported as *Gyrodinium aureolum*) eg.: sudden, rapid bursts of uncontrollable swimming followed by long periods of inactivity on the bottom of the aquaria, heaving of the operculum and mouth, small, rapid convulsing movements of the whole body, loss of orientation and white mucoid secretions protruding from the gill cavity.

Only occasional bursts of rapid movement were observed in fish in the lipophilic extract (Trial A) group with fish seeming to occasionally lose swimming orientation and "float" around in the aquaria. However, this seemed common in the controls also and may constitute a normal

behaviour. Although no mortality was seen in this group over the five day period, fish seemed to have a slightly laboured ventilation of the gills shown by large opercular and mouth movements. In contrast, animals in all the control groups seemed calm and unstressed with slow rhythmic breathing, and gentle swimming around the aquaria interspersed with inactivity on the sides and bottom of the aquaria.

Table 4.4: Number of fish mortalities exposed to whole-cell cultures, cell free (100%, 60%, 15%) culture medium and lipophilic extracts of *A. minutum* and pure GTX $_{(1-4)}$ toxins and their respective controls.

Trial	Treatment	Time exposed (hrs)	Number of fish exposed	Mortality
A	whole-cells	24	12	6
	cell-free culture	24	12	3
	lipophilic extract	24	6	0
	control	24	6	1
B	cell-free culture	2.5	6	0
	control	2.5	6	0
C	cell-free, 60% strength	10	9	3
	cell-free, 30% strength	10	10	4
	control	10	3	0
D	cell-free, 15% strength	144	6	1
	control	144	3	0
E	pure GTX (1-4) toxins	24	6	0
	control	24	2	0

Upon removal from their respective aquaria, many treated fish from Trials A, B, C and D were found to have large volumes of mucus associated with the gill cavity. Gills appeared necrotic in some instances but all seemed to have more secreted mucus than was observed in the controls. This seemed to be directly related to the concentration of *A. minutum* culture (whole-cell and cell-free) and the time of exposure. The higher the concentration of culture medium and the longer the exposure the more mucus was present on euthanasia. The skin and eyes appeared normal in colour and fins were entire with no apparent necrosis.

4.3.3.2.2 *Microscopic examination*

There was a wide degree of individual variation in response to exposure to the whole cell

culture and culture-filtrate of *A. minutum*. However, each exposure group exhibited the same pathological tissue damage the only difference between individuals and groups being the degree of severity. Typical pathology, when compared to the controls (Figs. 4.1 & 4.2), consisted of swelling of all respiratory epithelia (mild to severe) (Figs. 4.3 & 4.4, Table 4.5)), degeneration and sloughing of the respiratory epithelium, varying degrees of swelling, vacuolation and degeneration of the gill chloride cells (Fig. 4.5) and hypertrophy of mucocytes (Fig. 4.3). No other organ tissue appeared to be affected by the treatments and there was no such pathology observed in fish from either the control groups or the group exposed to lipophilic *A. minutum* extracts.

Trial A: Severe swelling of the respiratory epithelium (Figs. 4.3 & 4.4), vacuolation and degeneration of the chloride cells (Fig. 4.5) and exfoliation of the respiratory epithelium in some places, were all apparent in the gills of fish exposed to the whole cell culture and culture-filtrate of *A. minutum*. No such changes were noted in the gills of fish in the control treatments (Figs. 4.1 & 4.2). These effects ranged in individual fish from mild to very severe, particularly the swelling of the respiratory epithelium which, in one case, was approximately 13μm (Table 4.5) when exposed to the whole cell culture or culture-filtrate of *A. minutum* but was found to be approximately 1.0μm or less in the control and lipophilic extract exposed gill sections.

Microsporidian infection (a common fish parasite) of the liver was detected in one animal while focal necrosis and non-suppurative inflammatory foci of the liver was observed in another. There also appeared to be some minor changes in the intestinal tissue, mainly the presence of presumptive macrophages containing ingested material. These changes were common in most specimens as well as in controls. Antemortem blood clots in the heart ventricular tissue were present in some fish from both the whole cell and cell-free culture treatments (Fig. 4.6), but they were not present in hearts of any fish in either the control (Fig. 4.7) group or the lipophilic extract group (Fig. 4.14). All other tissue seemed unaffected by the whole cell culture and cell-free *A. minutum* medium, the tissues from which appeared very similar to the control tissue (Figs. 4.8-4.10). Similarly, no tissue, including the gills, appeared affected by the lipophilic extract treatment (Figs. 4.11-4.15).

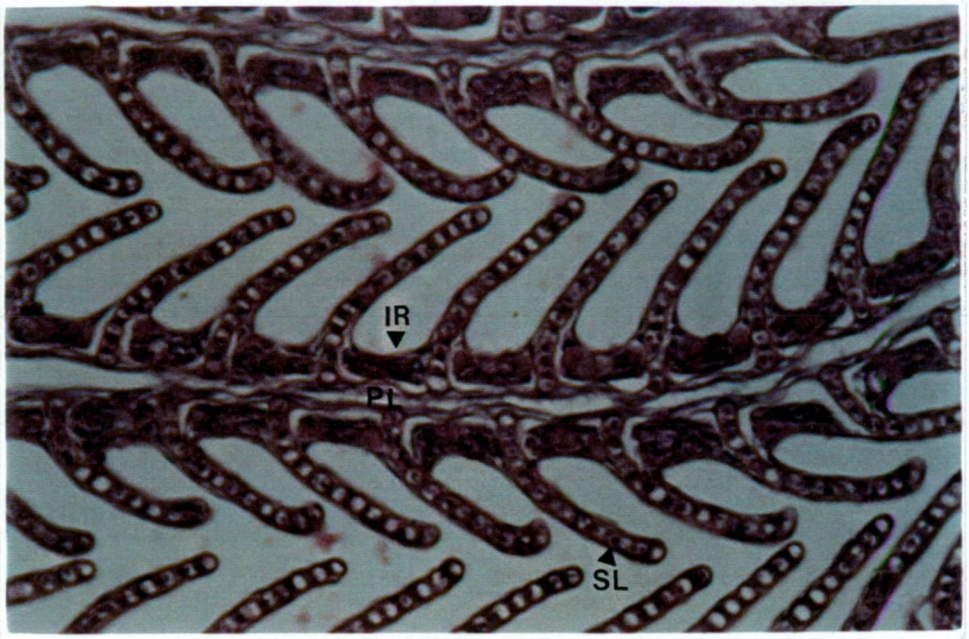


Figure 4.1: Gills of *R. taparina* exposed to clean seawater with the addition of GSe nutrients (control). H&E x 400, PL: Primary Lamellae, SL: Secondary Lamellae, IR: Interlaminar Region.

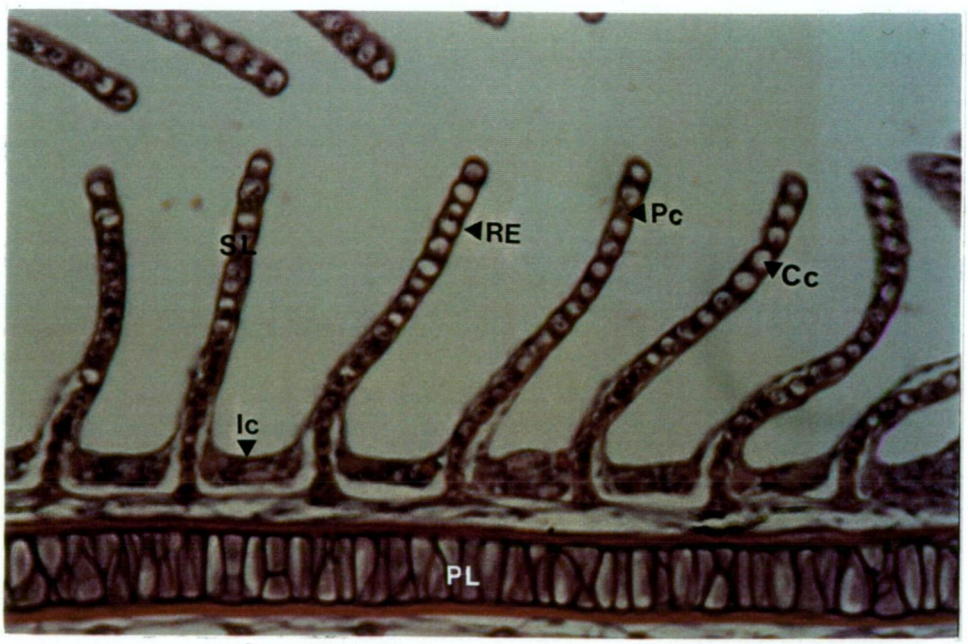


Figure 4.2: Gills of *R. taparina* exposed to clean seawater with the addition of GSe nutrients (control). H&E x 625, PL: Primary Lamellae, SL: Secondary Lamellae, RE: Respiratory Epithelium, Ic: Interlaminar cells, Pc: Pillar cells, Cc: Capillary channel.

Figure 4.3: Gills of *R. taparina* exposed to cell-free, *A. minutum* culture medium. H&E x 625, ▲: severely swollen respiratory epithelium, Δ: epithelial cells pushed into interlaminar region, ⁕: swollen mucocytes.

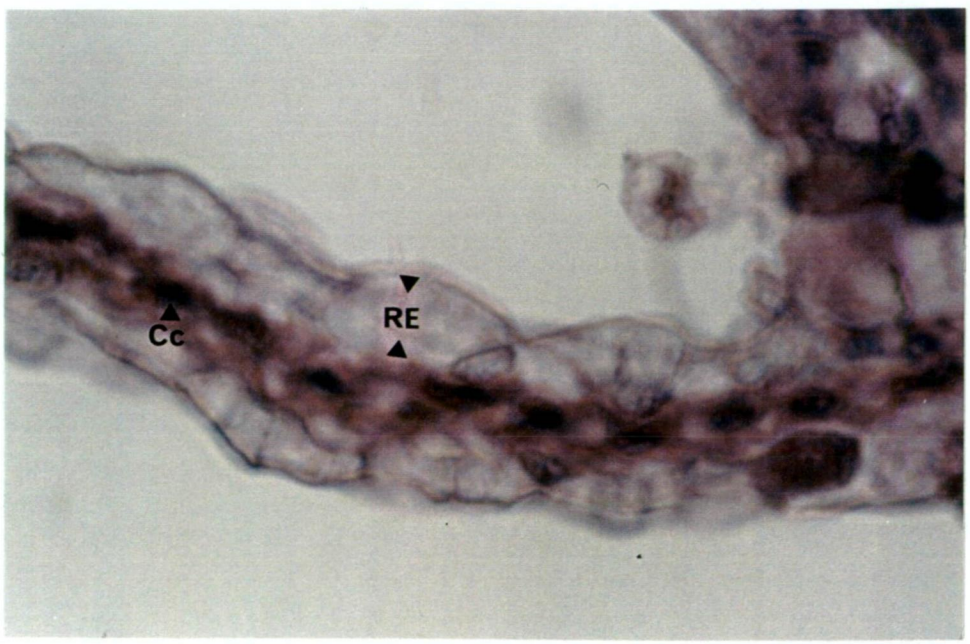


Figure 4.4: Single secondary lamella of gill from *R. taparina* exposed to whole cell culture of *A. minutum*. H&E x 1250, RE: swollen Respiratory Epithelium, Cc: Capillary channel.

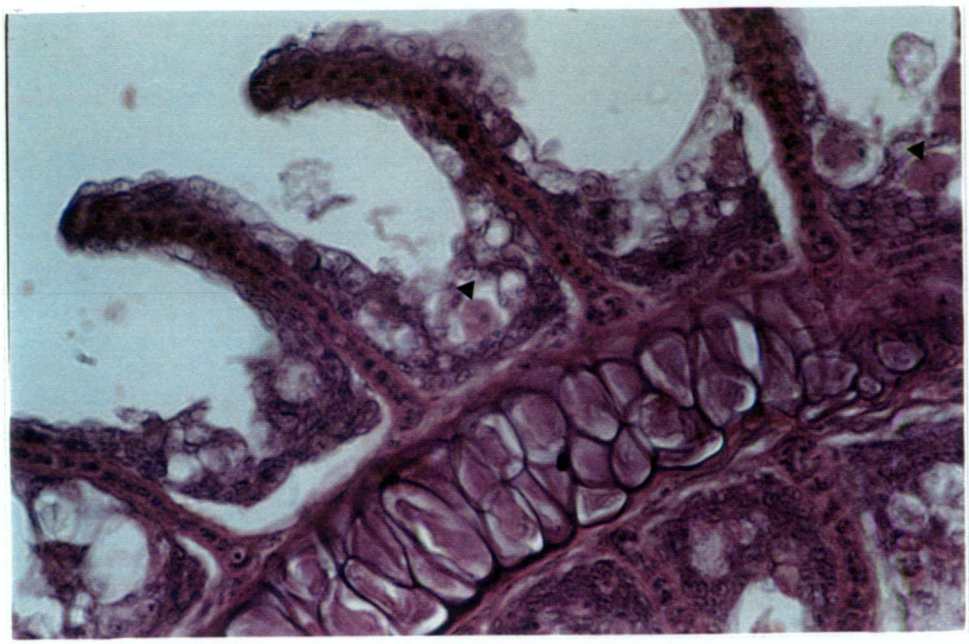


Figure 4.5: Gills of *R. taparina* exposed to cell-free, *A. minutum* culture medium. H&E x 625, ▲: vacuolated chloride cells with the appearance of pycnotic (condensed) nuclei.

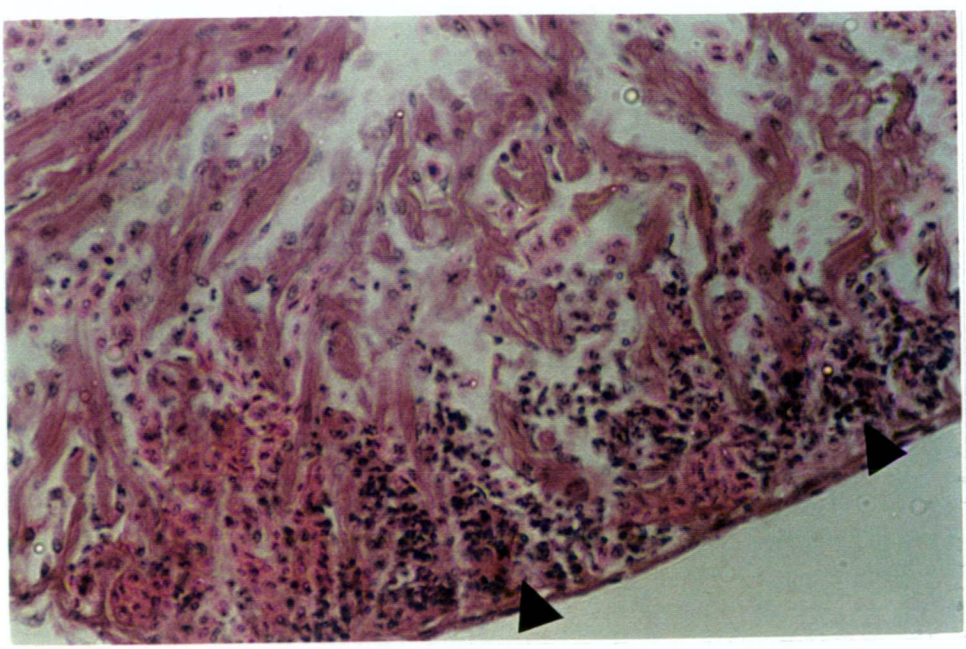


Figure 4.6: Heart tissue from *R. taparina* exposed to *A. minutum* whole cell culture. ▲: antemortem blood clot (dense erythrocytic aggregations). H&E stain x 625.

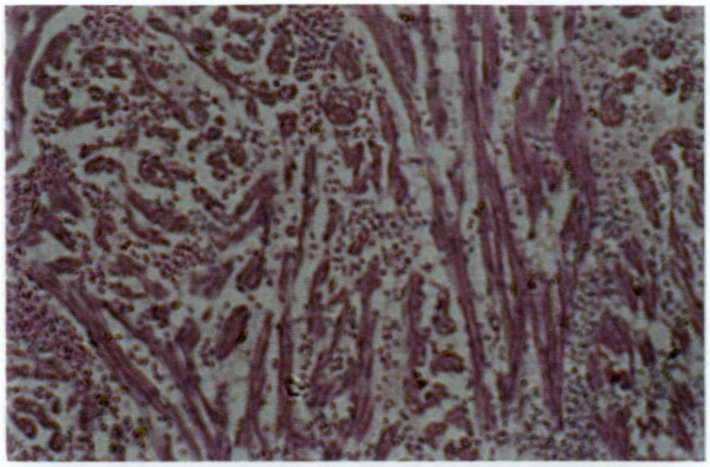


Figure 4.7: Heart tissue of *R. taparina* exposed to clean seawater with the addition of GSe nutrients (control). H&E stain x 250.

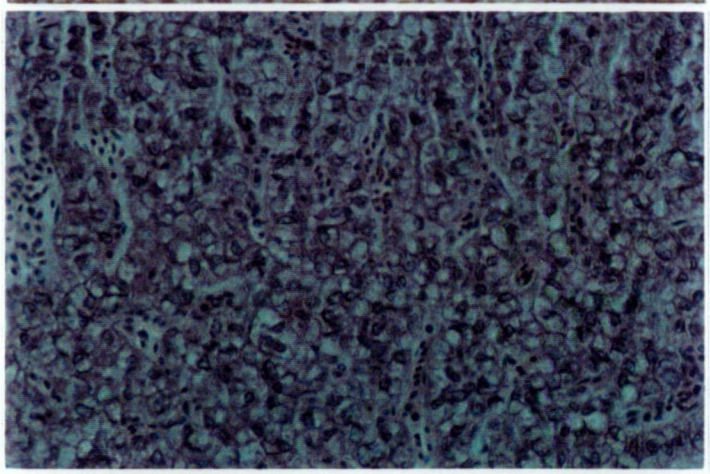


Figure 4.8: Liver tissue of *R. taparina* exposed to clean seawater with the addition of GSe nutrients (control). H&E stain x 625.

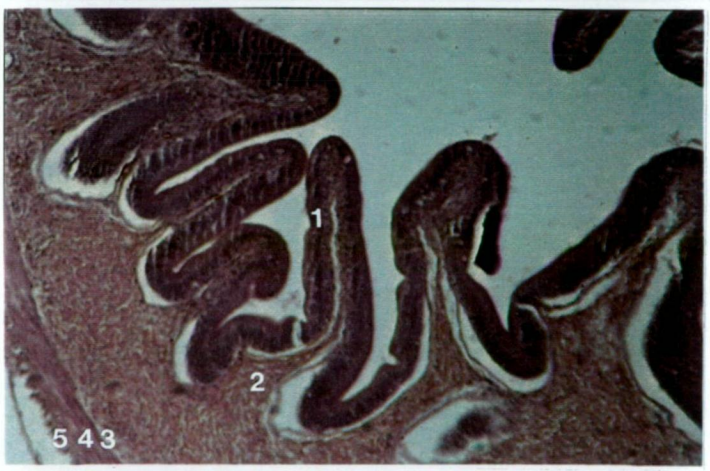


Figure 4.9: Anterior intestinal tissue of *R. taparina* exposed to clean seawater with the addition of GSe nutrients (control). H&E stain x 157.5. 1: mucosal epithelium, 2: submucosal stratum (stratum compactum & stratum granulosum), 3: circular muscle layer, 4: longitudinal muscle layer, 5: outer membrane (tella subserosa & serous membrane).

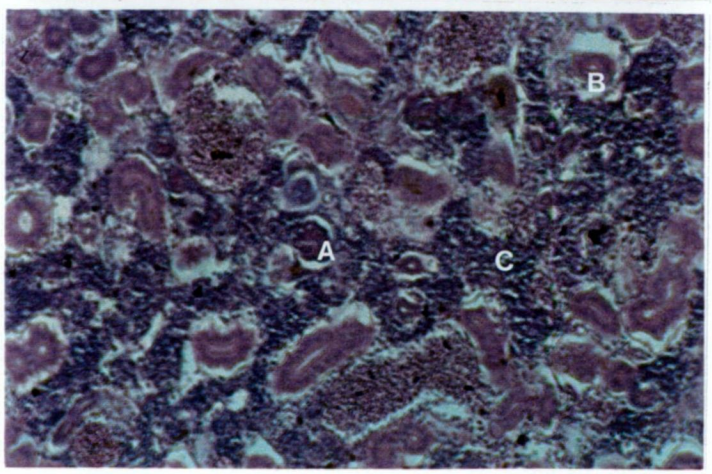


Figure 4.10: Kidney tissue of *R. taparina* exposed to clean seawater with the addition of GSe nutrients (control). H&E stain x 250. A: glomerulus, B: renal tubule, C. lymphoid tissue.

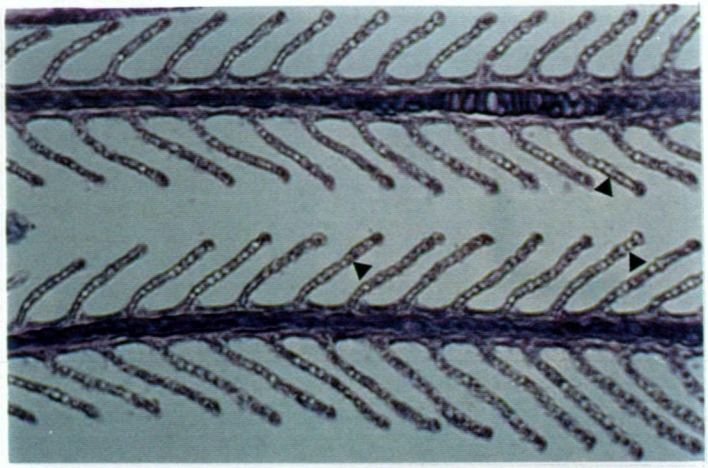


Figure 4.11: Gills from *R. taparina* exposed to a lipophilic extract of *A. minutum*. H&E stain x 250. ▲: normal thickness respiratory epithelium and the absence of lamellar fusion.

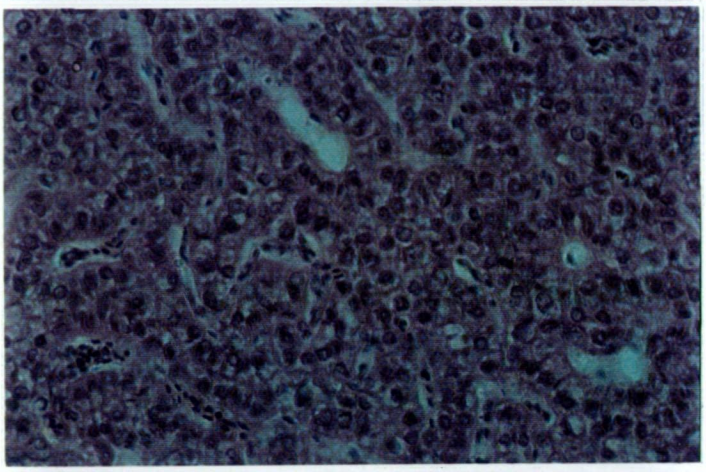


Figure 4.12: Liver tissue from *R. taparina* exposed to a lipophilic extract of *A. minutum*. H&E stain x 625.

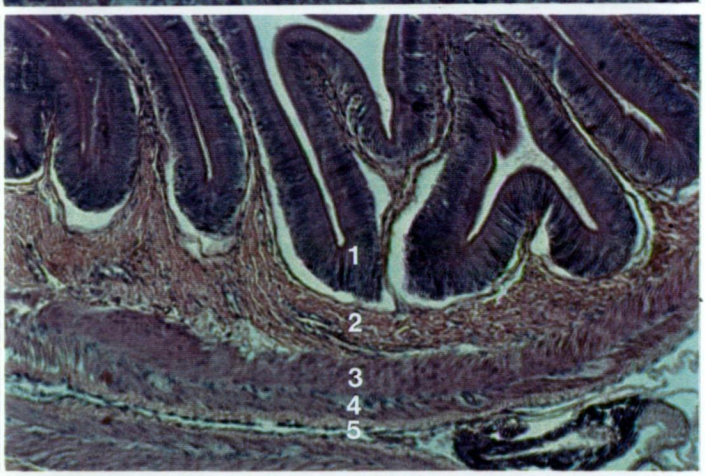


Figure 4.13: Anterior intestinal tissue from *R. taparina* exposed to a lipophilic extract of *A. minutum*. H&E stain x 250. 1: mucosal epithelium, 2: submucosal stratum (stratum compactum & stratum granulosum), 3: circular muscle layer, 4: longitudinal muscle layer, 5: outer membrane (tella subserosa & serous membrane).

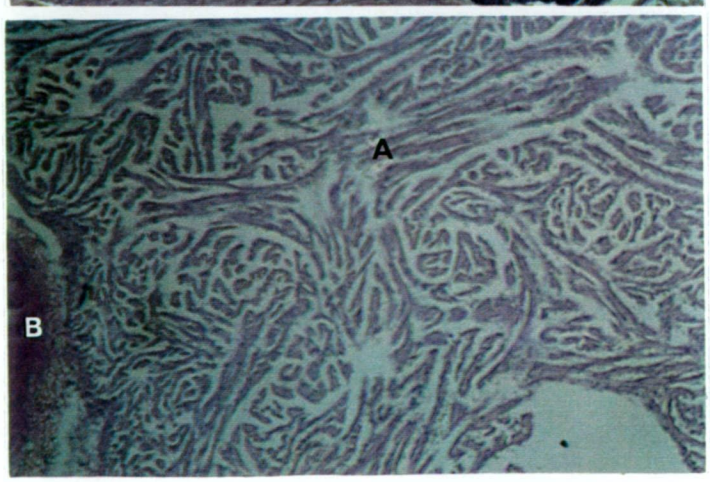


Figure 4.14: Heart tissue from *R. taparina* exposed to a lipophilic extract of *A. minutum*. H&E stain x 157.5. A: ventricle, B: bulbus arteriosus.

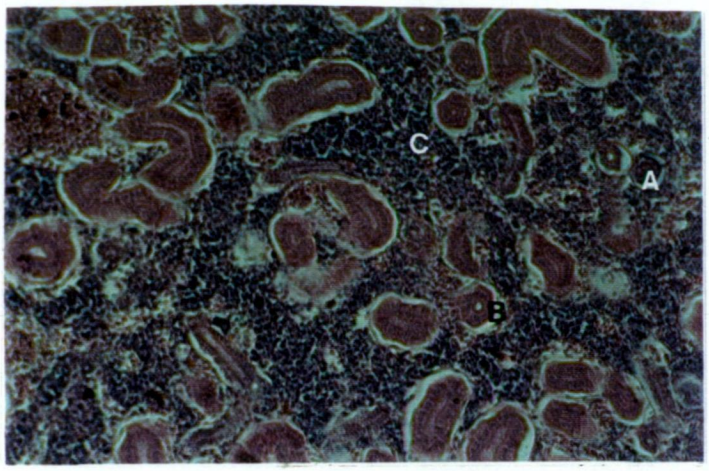


Figure 4.15: Kidney tissue from *R. taparina* exposed to a lipophilic extract of *A. minutum*. H&E stain x 250.A: glomerulus, B: renal tubule, C. lymphoid tissue.

Trial B: Marked swelling of the gill respiratory epithelium was noted in all of the fish exposed to cell-free *A. minutum* growth medium (Figs. 4.3 & 4.4). This ranged in severity from mild on some secondary lamellae to very severe in others.

Table 4.5: Thickness of respiratory epithelia (μm) (respiratory epithelial distance) of the gills in flounder exposed to whole cell culture and cell-free medium of *A. minutum*. Data represents averages of four measurements per secondary lamellae on nine primary lamellae of two gill arches. This data has also been calculated as a total average per fish.

***A. minutum* Treatment**	**Respiratory Epithelial Distance (μm)** arch 1	arch 2	Average per. fish
whole cell culture			
1	9.86±3.43	10.56±2.95	10.21±3.16
2	11.22±3.70	11.64±4.97	11.36±3.56
3	11.88±4.34	10.48±3.88	11.18±4.34
4	9.40±3.65	9.20±3.71	9.30±3.66
5	12.12±3.55	13.16±4.05	12.64±3.82
cell-free medium			
1	6.21±1.50	7.10±2.42	6.65±2.05
2	7.26±1.95	7.76±2.17	7.51±1.13
3	7.08±2.49	7.26±2.29	7.17±2.29
4	8.45±2.90	8.49±3.01	8.47±2.93
5	8.34±2.95	6.38±2.17	7.36±2.76
6	9.37±3.36	10.53±3.34	9.63±3.36
7	11.33±5.58	12.28±5.35	11.80±5.45
8	7.04±3.54	6.34±2.70	6.69±3.14
9	7.62±4.05	4.70±1.25	6.16±3.32

However, in all treated fish there was some epithelial swelling evident to some degree.

Accompanying the swelling was some cytoplasmic shrinkage in the gill chloride cells (Fig. 4.5) and patchy epithelial exfoliation. Mild hyperplasia of the gill epithelial cells was also noted on some secondary lamellae but only at the distal portions. All other tissues appeared unremarkable and were similar to the controls (Figs. 4.7, 4.8-4.10). The numbers of mucocytes were also counted on alcian blue stained secondary lamellae (Fig. 4.16) of both exposed and control fish however, no statistical difference could be found between them.

Trial C: Marked respiratory epithelial swelling and some degeneration of the epithelia of the gills of fish exposed to 30% strength *A. minutum* cell-free medium was noted (see Fig. 4.3 & 4.4). This swelling was accompanied by epithelial exfoliation and lifting of the basal

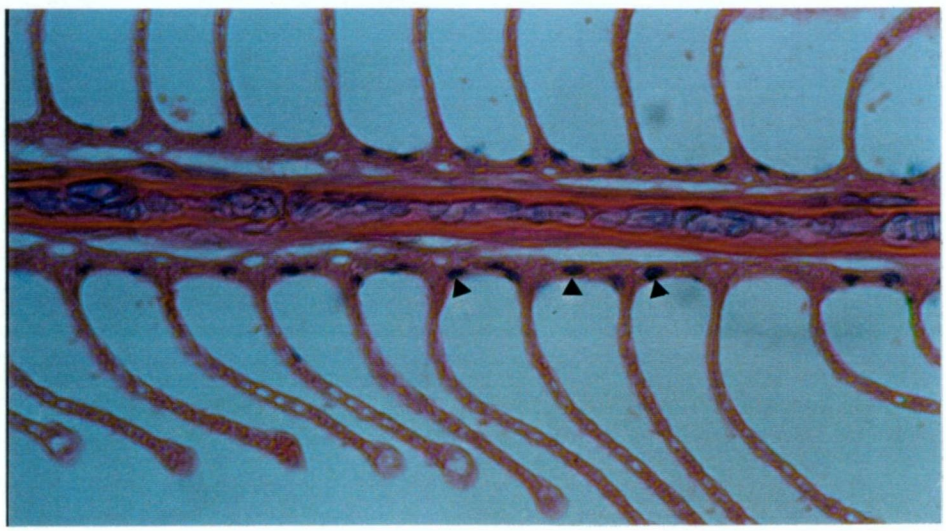


Figure 4.16: Mucocytes (stained blue with alcian blue: ▲) on the secondary lamellae and in the interlaminar region of gills of *R. taparina*. E&Alcian Blue x 250.

lamina in some cases while cytoplasmic shrinkage in the gill chloride cells was common (see Fig. 4.5). There also appeared to be mild hyperplasia of the gill epithelial cells particularly on the distal portions of the secondary lamellae of some fish. Antemortem blood clots were observed in the heart (ventricular) cavity of many fish from both 30% and 60% treated groups, which exhibited aggregations of erythrocytes with degenerate nuclei (see Fig. 4.6). Some leucocytes were present at the time of clot formation with others possibly migrating later. Such clots were not observed in the control fish. In general, all other tissues where unremarkable in their histology, as were all tissues including gills from the control fish, and did not seem affected by the *A. minutum* cell-free medium.

Compared to the gill pathology observed in Trial A and the 30% exposure (Trial C), there was minor gill damage in fish exposed to 60% cell-free *A. minutum* medium. Epithelial swelling was minimal as was lifting of the epithelium. Where swelling was noted it was confined mainly to the distal portions of the primary lamellae. No remarkable changes in chloride cells or interlaminar cells were observed. Antemortem blood clots where again apparent in the heart resembling those in the 30% treatment (see Fig. 4.6). All other tissues where similar to the control tissues (see Figs. 4.8-4.10).

Trial D: Mild epithelial swelling was apparent in all gills exposed to 15% cell-free *A. minutum* medium (see Fig. 4.3). The swelling seemed mainly confined to the distal portions of the secondary lamellae. This exposure continued for six days and as a consequence of the sublethal, chronic nature of the exposure, some minor fusion of secondary lamellae was also apparent with some very early epithelial hyperplasia. Fusion of secondary lamellae may require up to several days of exposure to become apparent (B. Munday: pers. comm.). Gill chloride cells seemed normal as did all other tissues when compared to the controls. There were prominent microsporidian aggregates observed in the livers of several treatment and control fish with one control fish exhibiting fatty degeneration of the liver. Antemortem blood clots were again visible in the heart ventricular cavity (see Fig. 4.6) while there was only slight autolysis of the intestinal epithelium. No remarkable pathology was observed in any other tissue which were similar to the control group (Figs. 4.8-4.10)

Trial E (*Pure GTX exposure*): Fish (30mm) where exposed to pure GTX toxins (97nm STX equiv.) for a period of 24 hours. No pathological tissue damage or change was observed in any tissue (Figs. 4.17-4.21). The gills appeared normal with evenly spaced and regular secondary lamellae covered by a thin and entire respiratory epithelium that had not exfoliated from the underlying pillar cells (Fig. 4.17). Chloride and mucous cells appeared normal. Slight, postmortem autolytic changes were apparent in the intestinal epithelium but these where minor and also observed in the control tissue. This was most likely due to enzymatic digestion of the intestinal epithelium after euthanasia as no fixative was introduced directly into the intestinal lumen. Liver (Fig. 4.18), intestines (Fig. 4.19), heart (Fig. 4.20) and kidney (Fig. 4.21) tissue all appeared normal when compared to the controls. No mortality was observed in the *Artemia* when exposed to the seawater containing the pure GTX_1-GTX_4 toxins and no mortality was observed in the control.

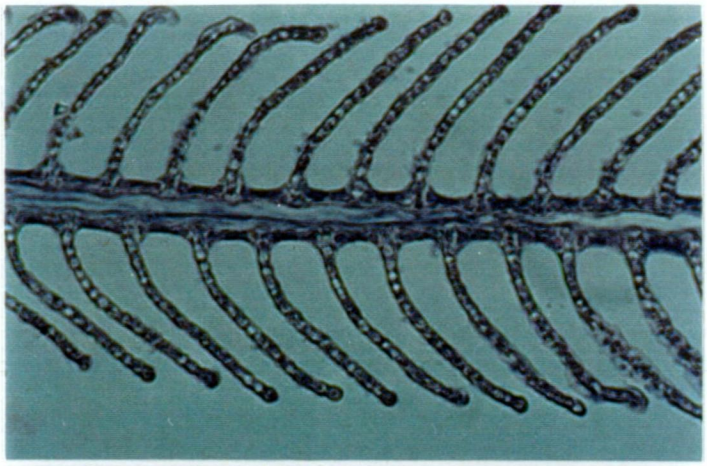


Figure 4.17: Gills from *R. taparina* exposed to pure GTX toxins (1-4) in the same molar percentage ratio as found in the Australian strain (AMAD-06) of *A. minutum*. H&E stain x 320.

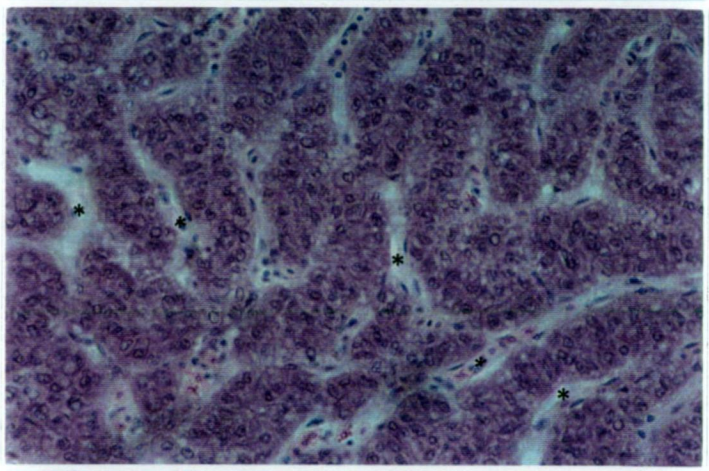


Figure 4.18: Liver tissue from *R. taparina* exposed to pure GTX toxins (1-4) in the same molar percentage ratio as found in the Australian strain (AMAD-06) of *A. minutum*. H&E stain x 625. ∗: sinusoid lumen.

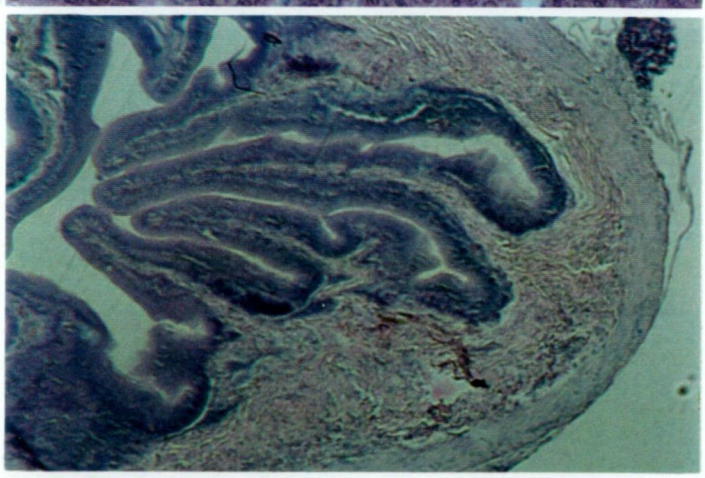


Figure 4.19: Anterior intestinal tissue from *R. taparina* exposed to pure GTX toxins (1-4) in the same molar percentage ratio as found in the Australian strain (AMAD-06) of *A. minutum*. H&E stain x 157.5.

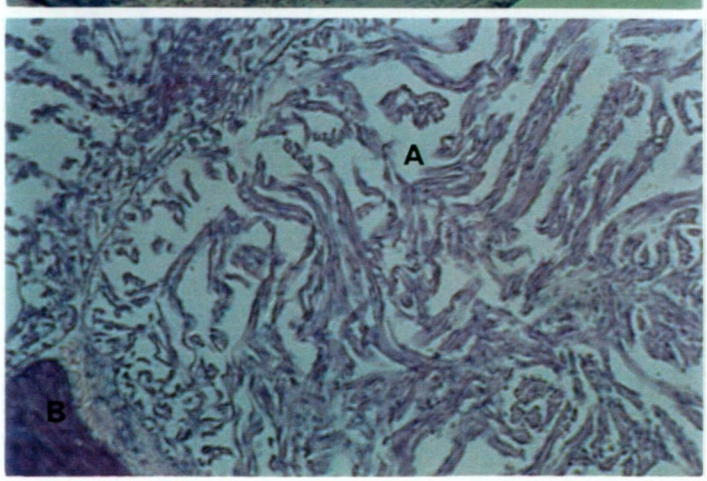


Figure 4.20: Heart tissue from *R. taparina* exposed to pure GTX toxins (1-4) in the same molar percentage ratio as found in the Australian strain (AMAD-06) of *A. minutum*. H&E stain x 250. A: ventricle, B: bulbus arteriosus.

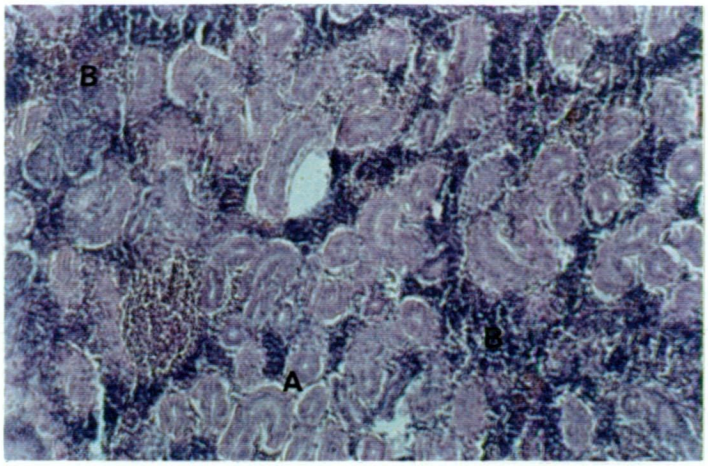


Figure 4.21: Kidney tissue of *R. taparina* exposed to pure GTX toxins (1-4) in the same molar percentage ratio as found in the Australian strain (AMAD-06) of *A. minutum*. H&E stain x 250. A: renal tubule, B. lymphoid tissue.

4.3.4 Discussion

The whole cell and culture and cell-free medium of *A. minutum* caused histopathological changes in the gill tissue of flounder. Ichthyotoxicity of algal culture media has been observed previously in many species of phytoplankton, particularly the Prymnesiophyceae, Raphidophyceae and Cyanophyceae however, it is not as commonly observed in the Dinophyceae. Chang *et al.* (1990) observed degenerative changes in the gills of chinook salmon such as swelling of the respiratory epithelium, mucus discharge and separation of the secondary epithelium from the pillar cells by a lamellar oedema fluid on exposure to *H. akashiwo*. Likewise, Roberts *et al.* (1983) observed changes in the gills of rainbow trout exposed to the dinoflagellate *Gyrodinium aureolum*, while Doi *et al.* (1981) and Shimada *et al.* (1983) have reported similar changes in the gills of yellowtail (*Seriola quinqueradiata*), but with added pathological changes in chloride and mucous cells when exposed to *Gymnodinium mikimotoi* and *Chattonella antiqua*, respectively. The genus *Alexandrium* (Dinophyceae) is becoming increasingly important in terms of research as it has been found to produce potent exocellular toxins (Ogata & Kodama 1986, Blanco & Campos 1988, Lush & Hallegraeff 1996) and also acid extractable hemolytic toxins (Simonsen *et al.* 1995). Thus far though, little work has been conducted to elucidate the chemical structure of these toxic substances.

The severe histopathological lesions produced in the flounder gills by the cell-free growth medium of *A. minutum* indicates that a toxic substance was present, it does not however indicate a specific chemical species. Mallat (1985) in his statistical review of structural fish gill changes induced by waterbourne toxic substances, indicated that the classic signs of exposure to a toxin, exhibited by the gills of fish eg. changes in gill epithelium (lifting, necrosis,

chemical structure of these toxic substances.

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The raphidophycean flagellates *Chattonella marina* and *C. antiqua* have been implicated in massive fish kills in which the gill histopathology of dead fish (marked epithelial lifting and variable swelling of the respiratory epithelium) was similar but not identical to that reported here in flounder (little epithelial lifting but severe swelling of the respiratory epithelium) from exposure to *A. minutum* (Shimada *et al.* 1983, Endo *et al.* 1985, Toyoshima *et al.* 1985). Although these species produce neurotoxic breve-like toxins associated with the NSP syndrome, direct gill damage is thought to be the result of *Chattonella*'s production of highly reactive superoxide anion radicals (O_2^-) and hydrogen peroxide (H_2O_2) (Oda *et al.* 1992, Tanaka *et al.* 1994, Kawano *et al.* 1996, Ishimatsu *et al.* 1996). However, the production of these highly reactive and unstable molecules requires the presence of photosynthetically

active cells (Oda *et al.* 1992) and the reactive oxygen species remain in the water column only for very short periods (min). Therefore, free radicals cannot be the cause of the gill damage observed in this work with *A. minutum*. Although adverse gill effects were apparent from exposure to whole cell cultures of *A. minutum* they were also apparent when fish were exposed to culture-filtrate after the medium had been stored for short periods (h) at 4°C and long periods (weeks) at -17°C.

The toxic principle affecting the gills directly would not seem to be one of the gonyautoxins (GTX) already known to be produced by *A. minutum*, as the exposure with pure GTX's failed to produce any gill lesions comparable to those found in the cell-free exposures. Likewise, no mortality of *Artemia* was observed from bioassays using the same seawater containing GTX toxins that was used to expose the flounder. This negative toxicity to *Artemia* and flounder, of GTXs when dissolved in the seawater, is supported by literature observations that indicate that PSP toxins only exert their primary, neurotoxic effect when consumed either as a consequence of feeding directly on the toxic alga by planktivorous organisms, or by an intermediate vector such as zooplankton (White 1981, White *et al.* 1989) or shellfish (Hallegraeff,1989, Oshima *et al.* 1993) that have sufficiently accumulated and magnified the concentration of PSP toxins within their own tissues. The negative result of *Artemia* exposure and flounder pathology when exposed to pure GTX toxins in terms of toxicity then asks the question of *what* component of the *A. minutum* culture-filtrate caused such severe changes in the gills of the flounder and the death of the *Artemia*.

4.4: Clinical pathological changes in fish blood chemistry and gill respiratory enzyme activity induced by *A. minutum* whole cells and cell-free culture medium

4.4.1 Introduction

Lesioning in major tissues is frequently mirrored by changes in physiology and metabolic processes. As with pathological changes in tissues, certain physiological changes may not be highly specific in identifying the particular toxic species present but, if viewed together, can be extremely useful in isolating the identity of a particular toxic type. Severe lesioning was observed in the gills as were antemortem blood clots in the cardiac muscle of flounder in this

study (section 4.3) exposed to *A. minutum* growth medium. Hence, physiochemical parameters of fish blood and gill tissue were measured in an attempt to further resolve the identity of the toxic substance(s).

4.4.2 Material and Methods

At the end of each experimental exposure (see Table 4.1) approximately 1.0ml of blood was taken from the gill arch of fish (in Trials B, C and D) using a 2.5ml sterile syringe. Blood was only taken from individuals of 100mm length or greater. Blood was immediately placed in a 5.0 ml Na^+ EDTA sequestrene tube (Johns) and placed on a slowly rotating (verticle) plate. Subsamples (approximately 10μL) were removed via capillary tubes and spun down for measurement of packed cell volume (PCV). The remaining blood was centrifuged to remove the cellular fraction and the plasma was assayed for osmolality (5500 VP Wescor osmometer), Na+, K^+, Cl- ions and plasma proteins, albumin and total globulins (Kodak Ektachem 250 AutoAnalyser).

4.4.2.1 Succinic dehydrogenase (SDH) enzyme activity

Succinic dehydrogenase (SDH) is a common cellular enzyme found exclusively in the mitochondrion. SDH catalyses the removal of two hydrogen atoms (in conjunction with FAD) from succinic acid to form fumaric acid (or step 7) in the tricarboxylic acid cycle and SDH is an integral part of the inner mitochondrial membrane (Eckert *et al.* 1988, Stryer 1988). Its activity gives an indication of cellular energy metabolism and, thus, the activity of the cell as a whole. Although common to all cells, in fish SDH is highly concentrated in the chloride cells of the gills. Gill chloride cells are rich in mitochondria as their primary role of osmoregulation requires large amounts of energy for active transport of ions (particularly sodium and potassium) against steep electrochemical gradients. Hence a change in osmoregulation will concomitantly be observed as a change in the activity of SDH.

SDH activity was assayed in fish in from Trials B, C and D according to a combination and modification of the methods by Clark & Porteous (1964), Sargent (1975) and Zaugg (1982). Fish were anesthetised in seawater containing 20 ppm benzocaine. Approximately 0.05-0.5g (wet weight) of gill filaments were trimmed from the supporting gill arches of one side of the anaethsitised fish and immediately placed in plastic Epindorf tubes containing 1.0ml chilled

homogenizing solution 1 (HS1) [0.3 moles L^{-1} reagent grade sucrose (102.7g L^{-1}); 0.02 moles L^{-1} Na_2EDTA (7.44g L^{-1}) and 0.1 moles L^{-1} imidazole (6.8g.L^{-1})], all adjusted to a final pH of 7.1 with HCl. Eppendorf tubes containing HS1 and gill filaments were then placed in ice with no loss of SDH activity for at least 3 hours (Zaugg 1982).

Homogenization of the filaments was then carried out within the Epindorf tube with a 5-10 second burst of a Polytron tissue homogenizer (PTH) and replaced in the ice or ice water. Distilled water (1.0ml) was then added to the homogenate and mixed. All diluted homogenates were then centrifuged at 6000 rpm for 7min, after which the supernatants were discarded and all tubes inverted for a short time to drain. All pellets were then thoroughly resuspended in 1.0ml homogenising solution 2 (HS2: HS1 containing 0.1g $100ml^{-1}$ sodium deoxycholate) with a 5-10 second burst of the PTH, giving a final protein concentration of between 1 and 3mg ml^{-1} (Zaugg, 1982). The resulting homogenates were then centrifuged as before but for 6 min and 0.1ml aliquots of the supernatant taken and placed in triplicate into the wells of an ELISA micro titre plate (reaction plate) and kept on ice.

Reagents were then added to the supernatant aliquots consisting of 0.06ml of sodium succinate (0.5M) and 0.04ml p-iodonitrotetrazolium (INT) violet (0.1% w/v) as an electron acceptor (Sargent *et al.* 1975). The reaction plate was then removed from the ice and incubated at room temperature. Optical density of the reaction solution was measured (492nm) at 15, 20 and 30 min by a elisa plate reader (MS 212). As the reading of all wells in this instrument was almost instantaneous it was found unnecessary to terminate the reaction with trichloroacetic acid (Clark & Porteous 1964, Sargent *et al.* 1975) which can affect absorbance readings greatly. The assay was standardized with INT-formazan from a stock solution of 23.6mg L^{-1} in 95% ethanol, to give final formazan concentrations equivalent to 0.01, 0.05, 0.10, 0.15 and 0.20 µmoles/sample (Clark & Porteous 1964).

4.4.2.1.1 *Protein determination*

Total protein was determined by the Lowry Method (Lowry *et al.* 1951). Ten µL of HS2 was placed into an Epindorf tube containing 990 µL of 0.5M NaOH. Then, 200µL of this diluted homogenate was then added to a test tube with a further 800µL of 0.5M NaOH. A blank was also prepared consisting of 1.0ml of 0.5M NaOH. Five ml of freshly prepared copper reagent

[containing 1 volume each of 1% w/v copper sulphate solution (2.5g $CuSO_4.5H_2O$ in 250ml distilled water), 2% w/v potassium sodium tartrate solution (5.0g $KNaC_4H_4O_6.4H_2O$ in 250ml distilled water), and 100 volumes 2% w/v sodium carbonate solution (40g anhydrous Na_2CO_3 in 2.0L distilled water)] was then added to all samples, mixed thoroughly with a vortex mixer and left to stand for 10 minutes. After 10 minutes, 0.5ml of 1N Folin reagent (stock commercial reagent diluted 1:1 with distilled water immediately prior to use) was added to all tubes then mixed immediately with a vortex mixer. After the tubes had been allowed to stand for 30 minutes their absorbance was read at 750nm. The protein assay was standardized with bovine serum albumin from a stock solution of 100µg ml^{-1} and 0.5M NaOH to give final protein concentrations equivalent to 0, 20, 40, 60, 80, 100µg protein ml^{-1}. The standards were processed as for the protein assay. Final protein concentration (mg ml^{-1}) was calculated as (1/slope of standard curve) x (500/1000) x absorbance.

4.4.3 Results

4.4.3.1 Succinic dehydrogenase (SDH) activity of the gills

The activity of SDH in the gills of fish exposed to *A. minutum* culture-filtrate was only found to be significantly different in the 15% and 60% cell-free exposures (Fig. 4.22).

When exposed to 15% culture-filtrate for 144 hours the activity of SDH in the treatment fish was significantly higher (p=0.022 Student's t-test) than its respective controls. Likewise, the activity of the fish exposed for 10 hours to 60% cell-free media was again higher (p=0.0001, Student's t-test) than its respective control. Indeed the activity of SDH in the 60% exposed fish was more than 10 times as active as its respective control. The SDH activity of gills from the 30% *A. minutum* cell-free treatment were also found to be higher than their respective control however, not significantly so. Fish from the 100% *A. minutum* cell-free exposure were found to have gill SDH activities below 0.05µmoles formazan mg $protein^{-1}$ ml^{-1} min^{-1} that was not significantly different from its control.

These data indicate that SDH activity was variably increased in response to exposure to *A. minutum* culture-filtrate. An increase in SDH activity indicates a concomitant increase in energy metabolism and hence the activity of the gill chloride cells in general. Although there

was no increase in SDH activity in Trial B (100% exposure), even at 100% concentration of culture-filtrate, this is probably due to time also being a factor in the exposures. Trial B exposure time was only 2.5h and was likely not long enough for the large changes in cellular-enzyme activity.

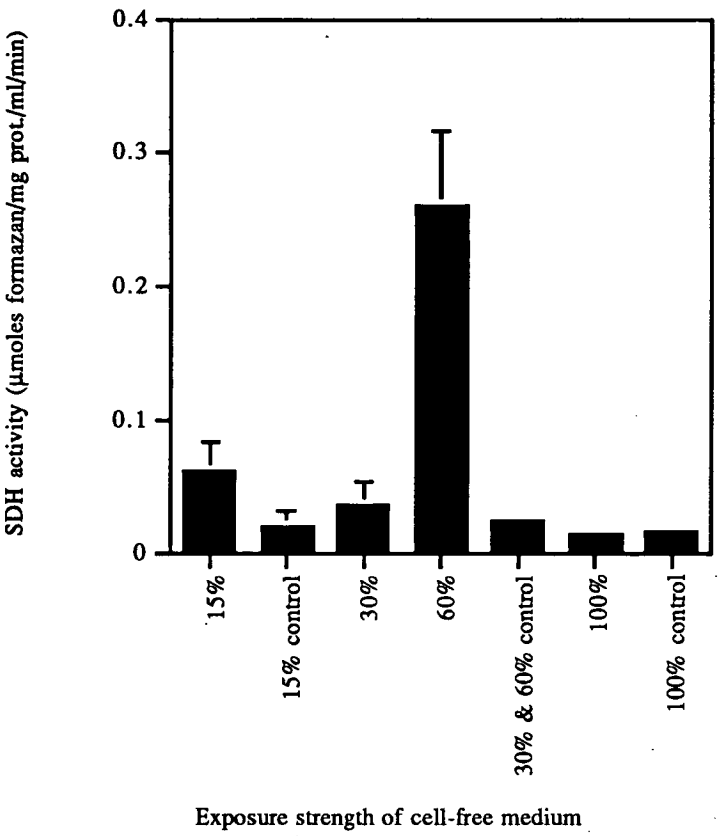

Fig. 4.22: Average activity (µmoles formazan mg protein^{-1}ml^{-1}min^{-1}) of the succinic dehydrogenase (SDH) enzyme from fresh gill tissue of fish exposed to different strengths of *A. minutum* culture-filtrate mixed with seawater containing GSe nutrients. For n see Tables 4.2 & 4.3.

4.4.3.2 Blood chemistry

Concentrations of chloride and sodium were slightly higher in all treatment trials (Table 4.6) when compared to their respective controls, however these elevations in concentration were not significant. No pattern could be observed in the concentrations of plasma proteins, again with no clear significant differences between treatments and controls.

Table 4.6: Concentrations of plasma monovalent ions (sodium, chloride) and plasma protein (albumins, globulins) in blood from fish exposed to various concentrations of cell-free *A. minutum* medium. For n see Tables 4.2 & 4.3.

Treatment	Exposure time (hours)	Na+ (mmol/L)	Cl- (mmol/L)	albumin proteins (g/L)	globulin proteins (g/L)
Normal		182.6 ± 15.1	164.8 ± 7.7	11.1 ± 2.02	11.0 ± 3.9
15%	144	162.0 ± 11.7	149.2 ± 5.9	13.4 ± 1.34	16.8 ± 2.2
15% control	144	145.7 ± 14.1	140.7 ± 3.1	13.0 ± 1.0	16.7 ± 3.5
30%	10	177.3 ± 5.9	162.3 ± 5.6	10.8 ± 2.1	11.5 ± 1.1
60%	10	171.2 ± 13.9	157.7 ± 15.7	12.2 ± 1.2	13.8 ± 2.8
30%/60% control	10	165.0 ± 6.1	156.0 ± 4.6	11.7 ± 3.5	14.3 ± 2.5
100%	2.5	164.0 ± 9.9	154.0 ± 10.0	11.2 ± 3.0	8.4 ± 3.4
100% control	2.5	157.0 ± 3.7	149.4 ± 2.7	11.0 ± 2.8	7.4 ± 4.2

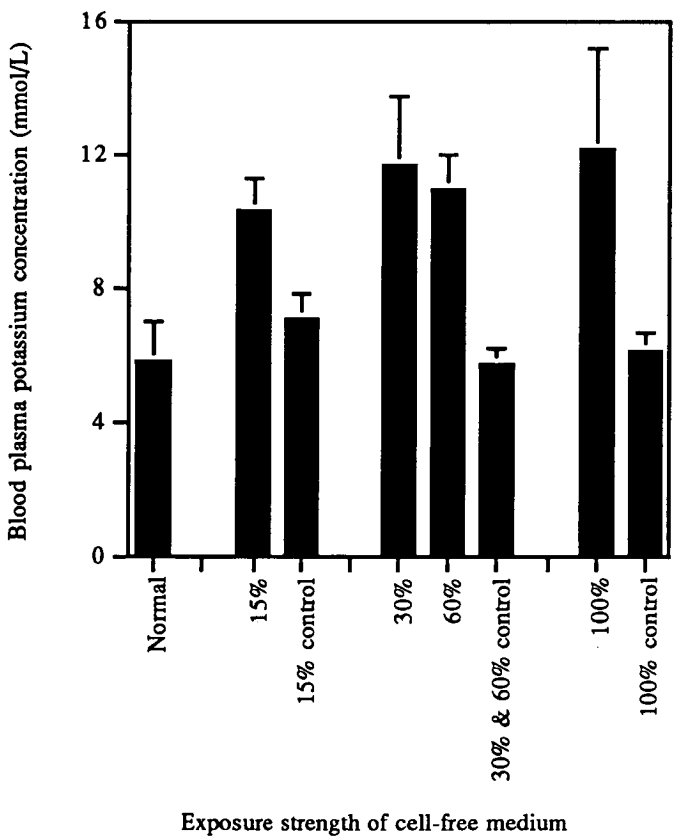

Fig. 4.23: Concentrations of blood plasma potassium in fish exposed to different strengths of *A. minutum* culture-filtrate mixed with seawater containing GSe nutrients. Also shown are normal levels of blood plasma potassium for this species. For n see Tables 4.2 & 4.3.

However, a significant difference (Students t-test exposure vs control: 100% Trial B, p = 0.002; 30% Trial C, p = 0.000; 60% Trial C, p = 0.000; 15% Trial D, p = 0.003) was found between the concentration of potassium in all treatment groups when compared to their respective controls. In all control groups, blood plasma potassium was low as is normal for this ion (Fig. 4.23), with the major portion of potassium being endocellular (Alberts *et al.* 1994), in this case within the erythrocytes. However, in all treatment groups the plasma potassium was high (Fig. 4.23) indicating a net loss of potassium into the blood plasma.

4.4.3.3 Plasma osmolality

All average plasma osmolalities of treatment fish were found to be higher than their comparative controls except for the 30% culture-filtrate treatment that had the same average osmolality as its comparative control (Fig. 4.24).

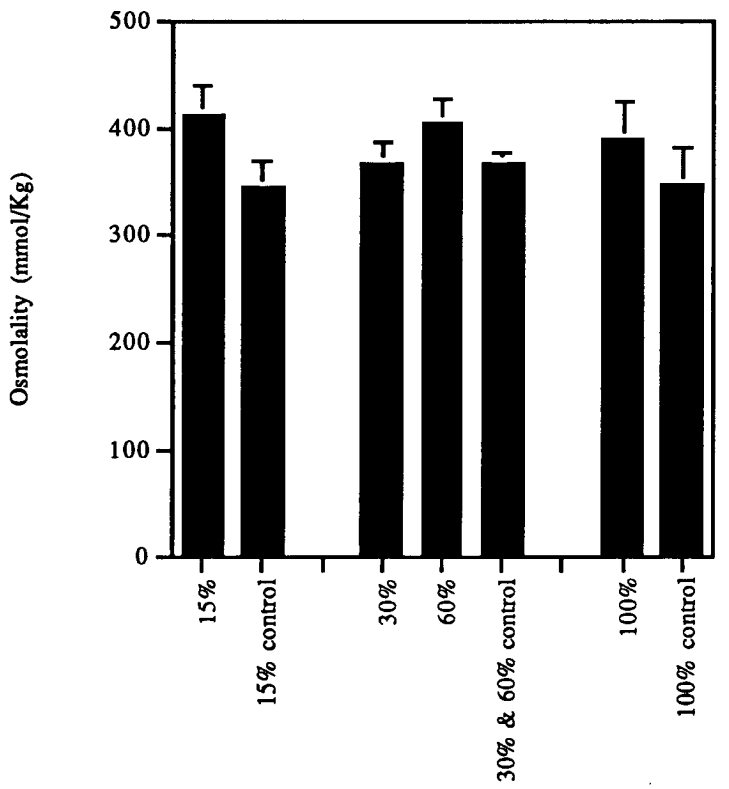

Figure 4.24: Average osmolality of blood plasma taken from fish exposed to different strengths of *A. minutum* culture-filtrate mixed with seawater. For n see Tables 4.2 & 4.3.

However, the only significant changes in osmolality were found in the treatment fish exposed to 15% cell-free *A. minutum* medium (p=0.015, students t-test) and the fish exposed to 60% cell-free *A. minutum* medium (p= 0.040, students t-test), when compared to their respective controls. Osmolality or osmotic concentration is an important attribute of body fluid. This depends largely on the concentration of solutes within the body fluid. While all solutes contribute to osmolality, small particles such as sodium, chloride and potassium ions are influential out of all proportion to their weight and account for over 90% of the osmolality of plasma. Changes in osmolality depend largely on the organs of osmoregulation. In the case of teleost fish this is the gills and the kidney.

4.4.3.4 Packed cell volume (PCV)

PCV is a simple test to determine the presence of anaemia (Dacie & Lewis 1975) through excessive loss of erythrocytes (red blood cells) or hemolytic anaemia and can also be used to detect haemoconcentration. PCV simply measures the ratio of the compacted (after centrifugation), cellular portion of a blood sample to the volume of the whole sample and is expressed as a percentage. As the cellular portion of the blood is mostly composed of erythrocytes it is thus a measure of erythrocyte concentration, which may give an indirect estimation of water loss.

All PCVs from fish in treatment groups were found to be lower than those for controls, however not significantly so. In contrast, for fish exposed to 15% culture-filtrate for 144 hours there was a significant reduction (p=0.007, Students t-test) in PCV at the end of the experimental period (Fig. 4.25). This indicated a general loss of erythrocytes from treatment fish's blood inducing a state of anaemia. This was particularly acute in the fish exposed for 144 hours to 15% cell-free, *A. minutum* medium, where the average PCV had fallen below 20%. It is possible that the non-significant reductions observed in PCV in the <24hr exposures where due to higher concentrations of cell-free media but at shorter exposure times indicating that time of exposure was an important factor. For a reduction in erythrocyte concentration either the erythrocytes themselves have to be affected (such as cellular lysis) directly or a retardation of their genesis.

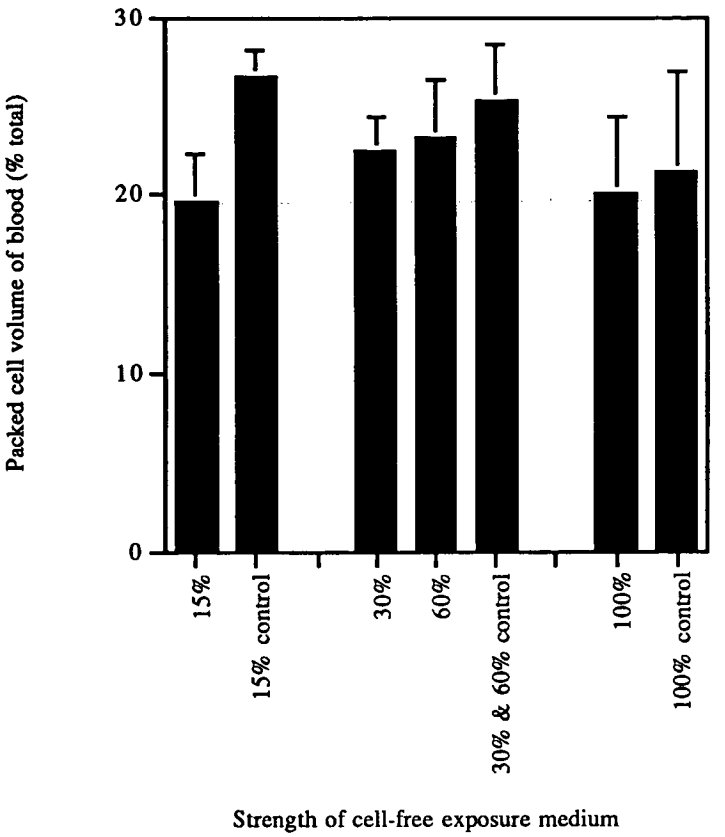

Figure 4.25: Average packed cell volumes of blood from fish exposed to different strengths of *A. minutum* culture-filtrate mixed with GSe seawater. For n see Tables 4.2 & 4.3.

4.4.4 Discussion

There appeared to be little disruption observed in plasma levels of sodium, chloride, albumin or the globulin proteins in the flounder exposed to the culture-filtrate of *A. minutum* and activity of the gill associated SDH enzyme was affected only in the short term. It is entirely possible that the osmoregulatory function of the gills was not affected enough (osmolality of plasma fluids was not significantly higher than the controls) by the lesioning and that the fish were able to compensate for any physiological perturbations with time. For example, changing the rate of active drinking, and the action of the chloride cells evidenced by changes in the rate of activity of the SDH enzyme, the fish were able to keep a relatively stable plasma osmotic concentration or at least keep within acceptable physiological parameters. Indeed, the fish were observed to be gulping with the mouth and operculum and although chloride cells were found to be

damaged this was not severe in many cases. What is puzzling though is the highly significant increase in plasma potassium concentration. Ionic potassium is compartmentalised in the bloodstream within the erythrocytes with very little free within the plasma, the ratio of endocellular/plasma potassium being approximately 30:1 (Eckert *et al* 1988). The accumulation of erythrocytic potassium, against it's steep electrochemical gradient, is due to two processes, the impermeability of peptide and protein molecules with negatively charged anionic sites (eg. carboxyls) in the cytosol, giving the endocellular medium a net negative charge requiring charge balance from positively charged ions; and the activity of the Na^+/K^+ ATPase pump (Eckert *et al.* 1988). The increases in plasma potassium observed here may have been due to erythrocytic fragility from small disturbances in plasma osmotic concentration, with subsequent leakage of potassium into the plasma. This would not be surprising given the damage observed to the gills. Also, localised damage to the gill tissues may have attributed to a general rise in plasma dissolved potassium in the blood. Decreases in PCV were observed in treatment fish when compared to the controls although these were small. It is equally possible that the culture-filtrate of *A. minutum* contained an unknown hemolysin that affected the erythrocytes directly. Some species of the Dinophyceae have been found to produce such hemolytic substances. Yasumoto *et al.* (1990) found hemolytic toxins (1-acyl-3-digalactosylglycerol and octadecapentaenoic acid) in *G.* cf. *mikimotoi* (reported as *Gyrodinium aureolum*) while other species of *Gymnodinium* have also been implicated in producing hemolytic toxins (Onoue *et al.* 1985, Onoue & Nozawa 1989, Hallegraeff 1991). *A. tamarense* has recently been observed to produce a powerful hemolytic agent, that was not a PSP toxin, in the exponential growth phase (Simonsen *et al.* 1995).

Hemolytic toxins are primarily cytotoxins as they disrupt cellular membranes (Rowe & Welch 1994).The primary effect of cytotoxins in fish normally takes the form of massive gill damage resulting in death of the fish. Speculatively, it is highly probable that the gill damage, small PCV reductions and raised potassium levels are connected, with the hemolytic effect being a coincidental cytotoxic effect from a toxin that affects cells regardless of tissue type. It is plausible to suggest then that *A. minutum* is producing a cytotoxic compound that has as yet only been reported anecdotally in the literature. This cytotoxin could have caused both the gill lesioning and some degree of hemolysis, both attributing to the observed increase in plasma potassium.

In nearly all cases where plasma potassium increases were observed, they were found to be at twice the level of the controls and the normal value of 5.82 ± 1.21 $mmolL^{-1}$. Raised plasma potassium concentration (hyperkalaemia) has serious complications causing cardiotoxicity, specifically changes in the QRS complex of electrocardiograms inducing shortening of the P-wave and some interval lengthening, bradycardia and atrial standstill which can lead to cardiac arrest (B. Munday, pers. comm.). Hyperkalaemia can also lead to neuromuscular disturbances (Guyton 1991, Greger & Windhorst 1996). In ideal conditions, potassium is accumulated in the endocellular medium while sodium is actively extruded into the exocellular medium via the energy (ATP) dependant Na+/K+ ATPase pump. The transmembrane gradient of potassium drives potassium back out of the cell, via potassium channels, into the exocellular medium creating an outside positive potential difference across the cell membrane (Greger & Windhorst 1996). Hence for most cells, potassium is of paramount importance for the generation and maintenance of cellular membrane potential. In terms of cardiotoxicity, high potassium in the exocellular fluid, as occurs in hyperkalaemia, causes a decreasing resting membrane potential in the cardiac muscle fibres. Very large quantities of potassium in the exocellular fluid can cause blockage of conduction of cardiac impulses from the atria to the ventricles via the atrioventricular bundles (Guyton 1991). So as the membrane potential decreases, due to increased exocellular potassium, action potentials of fibres also decrease as do the intensity of action potentials, resulting in a progressively weaker contraction and slower rate of contraction of the heart and in extreme cases, cardiac arrest. Greger (1996) and Sperelakis (1996) indicate that an elevation of potassium in the exocellular fluid to only twice its normal level, as observed here, can cause cardiac arrythmia and death. Such slowing of cardiac activity and thus circulation, could explain the appearance of the antemortem blood clots in the hearts of many of the fish exposed to the culture-filtrate of *A. minutum*. Levels of plasma potassium were found in nearly all cases to be twice that of the normal or control levels indicating that disruptions to the cardiac rhythm (no electrocardiographic studies were undertaken) were probably causing the observed clots. Also, in the longer exposures using higher concentrations of culture-filtrate sudden death was widespread and this was normally preceded by long inactive periods with high ventilation rates (observed as rapid opercular movements). A dramatic slowing of cardiac activity would have reduced circulation (oxygenated blood and nutrients) to all tissues, reducing overall activity and this would have been particularly exaggerated by the extensive damage to the gill further reducing available oxygen. Although, neurotoxins such as the brevetoxins produced by *Chattonella marina* have been found to also produce cardiac arrest in

red sea bream (*Pagrus major*) from depolarization of the vagal nerve (Endo *et al.* 1992), we have discounted this scenario for the gonyautoxins produced by *A. minutum* in this study as no effect was observed from pure gonyautoxins in both *Artemia* and flounder.

Another key function of potassium concentration, affected by hyperkalaemia is that of the maintenance of a stable cell volume. Most cells are freely permeable to water according to the prevailing osmotic gradient. Hence to maintain a constant cell volume an osmotic equilibrium must be achieved across the cell membrane. However, some metabollicaly active substances such as amino acids and glucose must be accumulated by the cell to carry out normal metabolic functions. To avoid swelling, the osmolarity caused by these substances must be balanced. This again is achieved by the activity of the Na+/K+ ATPase pump. The pump extrudes sodium in exchange for potassium creating an inward sodium gradient and an outward potassium gradient. Cells are normally poorly permeable to sodium but channels allow the permeability of potassium. The movement of potassium to the exocellular medium creates an outside positive potential which drags chloride from the endocellular to exocellular environment. Osmotically, the low endocellular chloride concentration outweighs the high endocellular concentration of organic solutes (Greger & Windhorst 1996). This cell volume equilibrium can be impaired by lack of a continual supply of metabolic ATP as the membrane is not completely impermeable to sodium. Also, it can be further disrupted by high exocellular concentrations of potassium as occurs in hyperkalaemia. High levels of exocellular potassium stimulate cellular uptake of potassium thus depolarizing the cell which in turn stimulates chloride uptake. Hyperkalaemia thus tends to swell cells and lysis can occur when swelling becomes extreme. Of course this does not happen in organisms that rely on the kidney for ionic and osmotic homoeostasis. However, marine teleosts such as the flounder rely on the gill for the removal of monovalent ions with the kidneys eliminating the divalent ions. The massive damage observed at the gill in the exposed flounder here may have impaired this gill function to such a degree that any form of cytotoxic activity (hemolysis) would have been exacerbated by the hyperkalaemic state of the animals.

4.5 General Discussion

Both damage of the respiratory epithelium of the gill and increased levels of plasma associated potassium could be explained by the presence of a hitherto unknown cytotoxin. The culture-

filtrate of *A. minutum* has shown itself, on every occasion, to be toxic to *Artemia* as well as the flounder. However, both the *Artemia* and the flounder were completely unaffected by the pure GTX toxins in the same composition and concentration that is normally found in this species of dinoflagellate. Indeed, one of the major contributors to forming hyperkalaemia is extensive cell death from cytotoxic substances (Greger & Windhorst 1996). One of the primary entry sites in fish for waterbourne toxicants is the gill with one of the first tissues being encountered being the blood. One would, with a high degree of certainty, expect then that the two tissue types to be primarily affected by such a cytotoxin would be the gills and the blood and this seems to be supported by the work presented here. As a consequence of this, a secondary effect was observed in apparent disruptions to cardiac functioning. This hypothesis seems to satisfy all effects that were observed although it does not move us any closer to recognising the actual toxin itself other than it does not appear to be one of the gonyautoxins (1-4) normally associated with this *A. minutum* isolate.

A. minutum's classic suite of neurotoxins, the gonyautoxins, are not known to produce any form of histopathological lesion, which also seems to be the case for the PSP toxin class as a whole (Hallegraeff: pers. comm). Nor is this group of toxins known to be actively excreted into the water column, although passive movement may occur at cell scenesenece when aged algal cells become "leaky". These two basic facts produce a dilemma for the results observed here. The toxicity of the culture medium of *A. minutum* is clearly apparent but does not reconcile as to the mode of toxic action (cytotoxicity) and the position (exocellular medium) of the toxic principle. Reasonably, we could postulate that *A. minutum* is producing another toxin, other than GTX's, that acts exocellularly with its toxic effect making it distinct from normal PSP neurotoxicity. The production of highly reactive free radicals (eg. O_2^-, H_2O_2) similar to those produced by species of *Chattonella* has been discounted as the toxic agent due to the requirement of living cells for this toxic, gill damaging effect to be observed. Gill damage was still apparent in this study upon exposure to culture-filtrate after the cells had been removed. Blanco & Campos (1988) found the culture-filtrate of a strain of *A. minutum* (reported as *A. lusitanicum*) to be toxic to *Skeletonema costatum*, *Pavlova lutheri* and *Isochrysis galbana* with all three being affected by water conditioned by the dinoflagellate. It was postulated that exotoxins in the medium killed the algal cells however, no specific toxic principle was suggested. Allelopathic tendencies have been found in other algal species such as the production of highly potent, exotoxic prymnesins by *Prynesium parvum* that inhibit the growth of several other algal species but did not inhibit the growth of the closely related *P.*

patelliferum (Johanssonl & Graneli 2000 in press). In contrast, a similar study indicated that culture filtrate of *A. minutum* was non-toxic to the growth of the non-toxic flagellate *Tetraselmis suecica* (Lush & Hallegraeff 1996).

Cytotoxicity is a common factor in many forms of cyanophytes (Armstrong *et al.* 1991; Gerwick *et al* 1994) affecting single-celled eukaryotic algal cells (Gromov *et al* 1991) and more complex, multicellular forms. Dinoflagellates are also highly important producers of ectocrine substances although little comprehensive exploration of their exact chemical nature and effects have been undertaken. *Gymnodinium mikimotoi* has been found to produce powerful cytotoxic and ichthyotoxic substances that are active in the exocellular medium of cultures of this alga (Partensky *et al.* 1989; Gentien & Arzul 1990; Gentien *et al.* 1991). Effects included marked damage to the digestive epithelium of juvenile bivalve molluscs and destruction of fish gill cells (Parrish *et al.* 1993). Yasumoto *et al.* (1990) isolated two compounds from the exocellular fluid of *G.* cf. *mikimotoi* (reported as*G. aureolum*) known to be hemolytic and ichthyotoxic while a wide range of toxic principles have been postulated to account for this exotoxic effect of *G.* cf. *mikimotoi*, most notably the presence of fat soluble toxins (Partensky *et al.* 1989) and free-fatty acids (Parrish *et al.* 1993; Smolowitz & Shumway 1997).

Some research has been conducted into the bio-enzymatic and chemical transformation of PSP toxins in marine organisms. This transformation process has been documented in species of bivalves such as the surf clam *Spisula solidissima* (Bricelj & Cembella 1995), the littleneck clam *Protothaca staminea* (Sullivan *et al* 1983) and the sea scallop *Placopecten magellanicus* (Cembella *et al.* 1994) but has not been reported from other organisms in the water column such as bacteria or other phytoplankton. The processes of transformation within some bivalves appear to range from simple epimerization to reductive desulfation and dehydroxylation, and even enzymatic decarbamoylation (Cembella *et al.* 1994). All of these tertiary metabolites of the PSP toxins and in particular the decarbamoyl and deoxydecarbamoyl toxins have as yet to be investigated as to their particular toxic potencies and effects. Also little is known if such transformations take place spontaneously within the water column or mediated by marine bacteria, in fact the fate of PSP toxins or whether they are actively excreted into the water column is as yet unknown but could represent a pool of unknown and unaccounted for toxins with a range of deleterious effects for both wild and cultured marine organisms alike.

Chapter 5: Endocellular and exocellular toxicity of *Alexandrium minutum* over its growth cycle

5.1 Introduction

Of the many genera of phytoplankton associated with causing harmful algal blooms (HABs) the dinoflagellate genus *Alexandrium* contains the largest number of toxic species (Anderson, 1998). Classically, species of *Alexandrium* have been documented as producers of the neurotoxic, PSP toxins, characterized by the STX parent molecule (see Fig. 1.1). There appear to be wide differences in the production of PSP toxins between different *Alexandrium* species (Anderson, 1990) (Table 5.1) and between isolates of the same species (Anderson *et al.*, 1990, Chang *et al.*, 1997) (Table 5.2). A noteworthy characteristic of *Alexandrium* and other PSP toxin producing dinoflagellates is that the total toxicity of a single isolate can vary dramatically under different growth conditions (Anderson, 1990, Plumley, 1997, Taroncher-Oldenburg *et al.*, 1997, Soames, Unpublished data) due mainly to changes in cellular toxin content but also toxin composition (Taroncher-Oldenburg *et al.*, 1997).

It is generally agreed among researchers that total toxin content of *Alexandrium* cells is highly variable over the cellular growth cycle with some differences occurring in the timing of maximal toxin production. Some researchers indicate that maximal toxicity of *Alexandrium* cells occurs in, or very close to, the exponential phase of culture growth, when cellular division is maximal (Boyer *et al.*, 1987, Cembella *et al.*, 1987, Anderson, 1990, Anderson *et al.*, 1990, Ishida *et al.*, 1993, Franco *et al.*, 1994, Franco *et al.*, 1995). Anderson *et al.* (1990), explain this correlation of cellular density and toxicity on the basis of the rate of toxin production required to replace "losses" to daughter cells during division. Anderson *et al.* further explain that the exception to this direct proportionality is when phosphorus is limiting in the medium and cellular division ceases due to nutrient limitation. With a halt in cellular division a dramatic increase in toxin production rate is observed. Proctor *et al.* (1975) and Boyer *et al.* (1985) indicate that suboptimal temperatures during growth cause increases in total cellular toxin concentration. However, White (1978) did not observe a direct relationship between toxicity and growth rate while observing increased toxin production with increasing salinity. The exponential phase-peak in total cellular toxicity is generally followed by a decrease in toxicity in species of *Alexandrium*, as the cells enter stationary or plateau phase (Boczar *et*

Table 5.1: Differences in PSP toxin profiles (mole %) between selected species and strains of the genus *Alexandrium*.

Species	Strain	STX	NEO	dc STX	GT X's 1	2	3	4	5	6	dc GTX2	dc GTX3	C1	C2	C3	C4	Source
A. catenella	O2	-	0.4	0.1	3.3	-	0.2	18.0	4.0	57.3	0.1	-	0.6	11.3	0.5	4.1	Hallegraeff et al. 1991
A. catenella	O9	-	1.0	0.1	3.9	0.1	0.4	26.2	1.9	30.4	-	-	1.0	21.2	2.3	11.4	Hallegraeff et al. 1991
A. fundyense	GTCA 29	8.0	28.0	-	>2.0	<2.0	8.0	4.0	<2.0	-	-	-	2.0	48.0	-	-	Teegarden et al. 1996
A. ostenfeldii	LF 37	-	-	-	-	0.6	0.1	-		91.6	-	0.1	7.7†	7.7†	-	-	Hansen 1992
A. ostenfeldii	LF 38	-	-	-	-	0.9	0.1	-		92.0	-	-	7.0†	7.0†	-	-	Hansen 1992
A. cohorticula	MMBS8811-3	23.0	-	-	2.6	2.0	5.5	36.8	9.1	-	-	-	2.2	7.5	2.5	8.8	Ogata et al. 1990
A. cohorticula	MMBS8811-1	23.0	-	-	3.8	3.9	10.2	44.5	13.3	-	-	-	0.5	0.8	-	-	Ogata et al. 1990
A. cohorticula	Chula 18	7.3	-	-	1.5	0.3	2.2	72.8	7.7	-	-	-	0.4	0.1	2.9	5.1	Ogata et al. 1990
A. cohorticula	Chula 8	0.4	-	-	1.1	1.3	8.1	60.8	7.2	-	-	-	1.4	2.9	1.9	15.0	Ogata et al. 1990
A. tamarense	LF2	2.0	3.0	-	<2.0Σ	14.0Δ	14.0Δ	2.0Σ	<2.0	-	-	-	82.0†	82.0†	-	-	Hansen 1989
A. tamarense	WH7	<2.0	11.0	-	5.0Σ	24.0Δ	24.0Δ	5.0Σ	4.0	-	-	-	56.0†	56.0†	-	-	Hansen 1989
A. tamarense	PLY 173a	17.0	73.0	-	-	-	-	-	-	-	-	-	10.0†	10.0†	-	-	Hansen 1989

Note: † = concentration is total of epimeric pair of C_1 and C_2

Δ = concentration is total of epimeric pair of GTX_2 and GTX_3

Σ = concentration is total of epimeric pair of GTX_1 and GTX_4

al., 1988). This decrease is normally attributable to CO_2 depletion in batch cultures (whether by pH or CO_2 shortage for photosynthesis in dense cultures) although older, senescent cells can become "leaky" to many substances, including toxins, with loss to the medium.

Table 5.2: Comparison of toxin composition profiles of different isolates of *A. minutum* (modified after Chang *et al.* 1997, Negri, Bolch & VanEmmerik unpublished).

Location (culture)	Toxin content (fm/cell)	Toxic potency (fg STX equiv. /cell)	Dominant toxins (mole %)				
			C1, C2	GTX1, 4	GTX2, 3	NEO	STX
New Zealand, Bay of plenty (AMABOP006)	22.27	11 590	0.0	7.6	4.0	77.2	11.2
New Zealand, Bay of plenty (AMABOP014)	11.61	6032	0.0	5.9	7.6	65.3	21.3
New Zealand, Marlborough Sounds (Anakoha A)	7.09	2360	0.0	23.0	36.0	13.0	28.0
New Zealand, Marlborough Sounds (Anakoha B)	4.86	1850	0.0	25.0	25.0	19.0	31.0
New Zealand, Tasman Bay (Croisilles A)	5.15	2280	0.0	51.0	9.0	22.0	18.0
New Zealand, Whangarei (NEPCC508)	2.76	880	0.0	96.4	2.9	0.0	0.7
Australia, Port River (AMAD 06)	2.54	1000	0.0	73.5	26.5	0.0	0.0
Australia, Port River (AMAD 15)	2.57	1000	0.0	82.9	17.1	0.0	0.0
Taiwan (AmTK1)	184.40	47 000	0.0	94.6	5.4	0.0	0.0
Taiwan (AmTK2)	79.50	20 600	0.0	99.6	0.4	0.0	0.0
Portugal, Laguna Obidos (NEPCC253)	1.16	370	0.0	97.4	2.6	0.0	0.0
Spain, Ria de Vigo (AL-1V)	18.00	6413	0.0	98.0	2.0	0.0	0.0
Spain, Ria de Vigo (AL-2V)	2.00	715	0.0	98.0	2.0	0.0	0.0
Spain, Ria de Vigo (AL-3V)	4.00	1383	0.0	94.0	6.0	0.0	0.0
France, Morlay Bay (AM89BM)	2.34	386	44.0	0.0	56.0	0.0	0.0
Australia, Newcastle (AMNC04, 05)	-	-	0.0	0.0	trace	trace	99.9

Non-nutritional variables such as temperature can elevate toxin production by the differential inhibition of pathways for toxin synthesis and cell division, or the increased availability of the toxic precursor arginine. The essential amino acid arginine has been implicated many times as a precursor of PSP toxin production (Anderson *et al.*, 1990, Shimizu *et al.*, 1990, Shimizu *et al.*, 1990, Plumley, 1997) (Fig. 5.1) and the highest rates of toxin production appear to result from an increased availability of arginine in the cell, due either to a lack of competition for this amino acid from pathways involved in cell division or an increase in *de novo* synthesis (Achituv & Bar-Akiva, 1973, Anderson *et al.*, 1990). Nutritional stresses (N, P and possibly C) also affect pathways for toxin synthesis but in different ways to those involving cellular division. These effects lead to the imbalances that are responsible for growth stage variability in batch culture toxin production.

Toxin composition of dinoflagellate isolates has been reported in the literature to be constant by

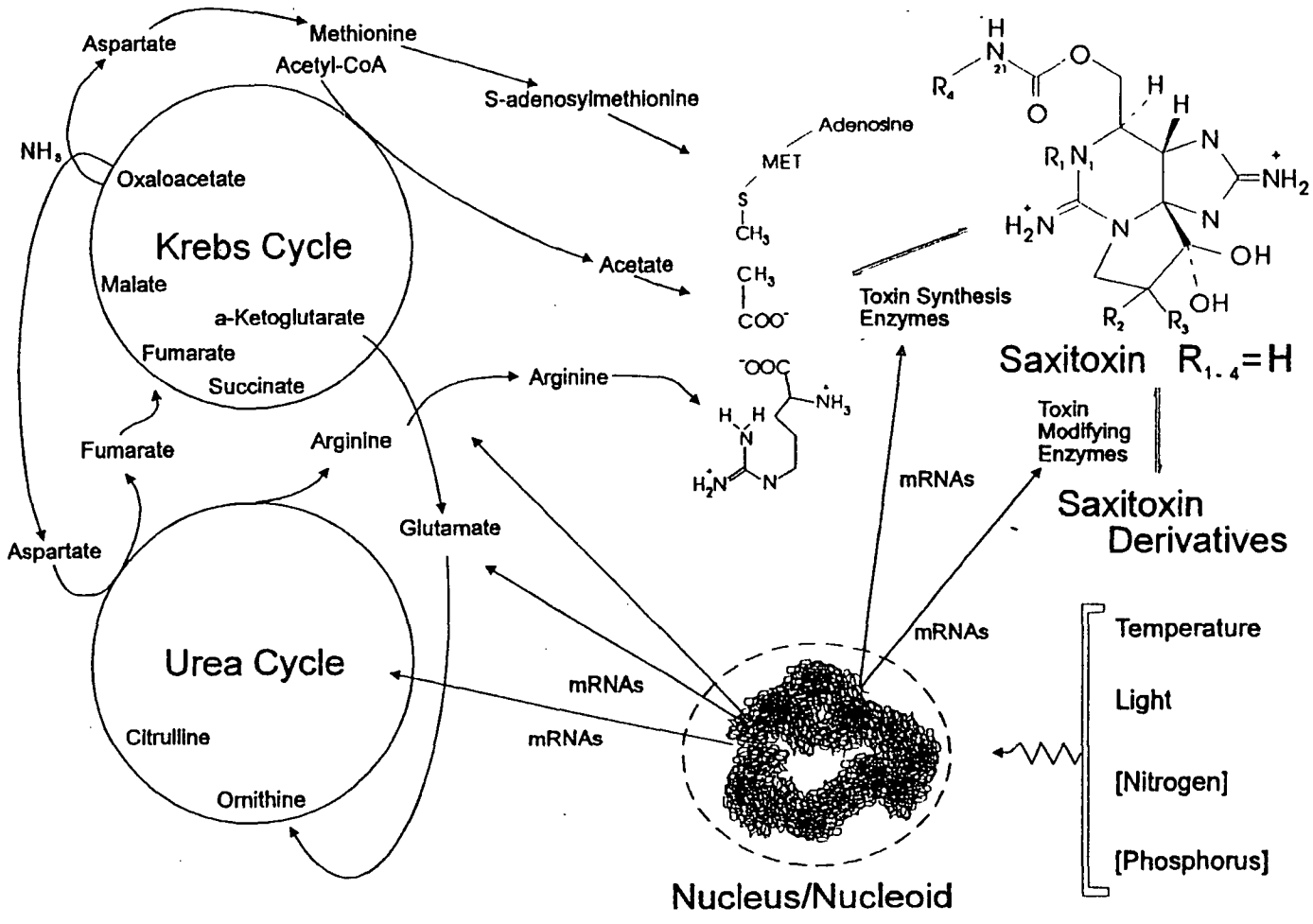

Figure 5.1: Proposed biochemical pathway of saxitoxin synthesis. Certain cellular metabolites are highlighted as growth of bacteria on these substrates can have substantial effects on toxin synthesis (after: Plumley 1997).

some workers (Boyer *et al.*, 1987, Cembella *et al.*, 1987, Ogata *et al.*, 1987, Ishida *et al.*, 1993, Sako *et al.*, 1995) and highly variable by others (Boczar *et al.*, 1988, Anderson *et al.*, 1990, Chang *et al.*, 1997, Taroncher-Oldenburg *et al.*, 1997). Some authors have attempted to use toxin profile as a phenotypic and taxonomic marker (Franco *et al.*, 1994, Franco *et al.*, 1995). Ogata *et al.* (1987) found no significant changes in toxin profile of a single *A. tamarense* (reported as *Protogonyaulax tamarensis*) strain (strain OF84423D-3) from Ofunato Bay, Japan, when grown under differing water temperatures and light intensities. Similarly, Cembella *et al.* (1987) found distinctive and constant toxin profiles in *Alexandrium* (=*Protogonyaulax*) isolates from widely separated geographical locations. Supporting this, Boyer *et al.* (1987) found dramatic effects on *total* toxin concentration in *A. tamarense* (=*P. tamarensis*) in response to nutrient limitation (N or P) but a constant (on a percent basis) toxin composition profile. Similarly, Sako *et al.* (1995) found that in over 70 isolates of *A. tamarense* and *A. catenella* the mole percentage of toxin composition did not change in any growth phase. In contrast however, Boczar *et al.* (1988) found variability in the toxin profiles of *A. tamarense* (=*P. tamarensis*) and *A. catenella* (=*P. catenella*) as cultures of these algae aged. In *A. tamarense* in particular the mole percent of neosaxitoxin increased substantially from 8-44% as total toxin levels per cell decreased, accompanied by a decrease in saxitoxin. In *A. catenella*, GTX_1 & GTX_4 (mole %) increased while that of GTX_2 & GTX_3 decreased.

Although some dinoflagellate species from the genus *Alexandrium* have been found to produce exotoxins (Hansen, 1989, Lush, 1993, Bagoien *et al.*, 1996, Lush & Hallegraeff, 1996) these toxic principles have never been proven to be PSP toxins. Indeed, it is difficult to ascribe a role to SCB toxins outside of the endocellular matrix. If we take the view of Boyer *et al.* (1987) that saxitoxin related SCBs are simply complex Nitrogen (N) stores (saxitoxin is 33% N on a molecular weight basis) then excretion of PSP toxins into the surrounding medium would have no purpose. Reductions in endocellular PSP toxins in batch culture stationary phase, may not only suggest leakage of toxins from senescent cells but also mobilization of N stores as the culture ages and N becomes limiting (Boyer *et al.*, 1987). It is well known that N is the most important nutrient limiting phytoplankton growth in marine, oceanic and coastal waters (Ryther & Dunstan, 1971, Chang & McClean, 1997). If PSP toxins where viewed as antiherbivory devices (Fiedler, 1982, Huntley, 1982, Ives, 1987) then again an exocellualr role cannot be imagined as PSP toxins only exert their primary neurotoxic and pharmacological effect when consumed, do not affect higher organisms when dissolved in seawater, and appear to have no

other activity than to bind with and block, membrane bound sodium channels (Oshima, pers. comm.). Although phytoplankton have been found to release up to 16% of their total fixed CO_2 as exudates (Larsson & Hagstrom, 1982) and algal (*Pavlova lutheri*) exudates comprise a proportion of the diet of some clams (*Venus verrucosa*) (Amouroux, 1984), conclusive evidence of the release of PSP toxins into the surrounding medium by dinoflagellates is lacking. However, Hu *et al.* (1995) have reported on two new water soluble Diarrhetic Shellfish Poisons (DSP) (DTX-5a & DTX-5b) (Table 1.1) that act as endocellular, water soluble storages of DSP in a less active sulphate form. They also postulate that this water-soluble form of DSP may be a means of excreting the lipid-soluble DSP acids and esters from the cell to the surrounding medium. Although such a mechanism has never been postulated for other phycotoxins including PSP toxins, it is by no means impossible. Implications of such molecular transformations are hard to quantify, however, changes in pharmacology and site of toxic action could be reasonably expected.

In light of the exocellular toxicity observed in *A. minutum* previously (Chapter 4), the following chapter attempts to determine the endocellular and exocellular toxicity of *A. minutum*, in batch culture, over the entire life cycle of the alga. Endocellular, PSP toxicity was determined by HPLC in terms of total toxicity (STX equivalents) and toxin composition (mole % of particular toxic fractions) using cells harvested at regular intervals from batch cultures. These results are correlated with toxicity of the exocellular medium from the same subsamples, in terms of SCB ability of the cell-free growth medium by neuroreceptor binding assays and total toxicity bioassays of the same cell-free growth medium when challenging the brine shrimp *Artemia salina.* Both endocellular and exocellular toxicities are discussed with a view to understanding the activity of PSP toxins in *A. minutum*'s life cycle and attempting to correlate endocellular PSP toxin reduction with the appearance of SCB toxicity in the exocellular medium and any possible connection between them.

5.2 Materials and Methods

5.2.1 Algal cultures

Four large 20L, polycarbonate (Nalgene) bottles were sterilized by autoclaving and filled to the 15L mark with clean, sterile (0.2μm filtered) seawater (33ppt). Sterile GSe medium was added

(Appendix 1) and the bottle shaken. *A. minutum* culture (50ml) in exponential growth phase was added to each of three bottles with one bottle (non-inoculated) remaining as a control. Sterile air was gently bubbled through all bottles using a standard aquarium pump connected to a sterilized filter unit (Activon, 0.22µm, 13mm). Cultures were allowed to grow for 35 days in controlled growth conditions (12hr light/12hr dark, 80µEm^{-2}s^{-1}, 17°C) before subsampling began. Sampling continued at regular intervals until the culture began to decline into the death phase after the stationary phase.

5.2.2 Cell counting and sample preparation

After 14 days, 100ml subsamples were removed from the batch cultures, every second day for 48 days then every 3-4 days for the remainder of the experimental period. All sampling took place at midday. Air bubbling in the bottles was increased slightly for a short burst (1 min.) to facilitate gentle cell mixing prior to subsampling and all subsamples were taken with the aid of a tap attached to the bottles. A subsequent 4ml aliquot was taken from the subsample which was fixed with glutaraldehyde to a final concentration of 2.0%. The fixed sample was used for cell counting in a Sedgwick-Rafter counting chamber.

The remaining subsamples were gently gravity filtered through GF/F filters (0.45µm pore size). The filtrate was removed and stored at 4°C to the end of the experimental period and then analysed for sodium channel binding ability with a competitive neuroreceptor binding assay (see sections 5.2.3.1 and 5.2.3.2), and a neuroblastoma tissue culture assay (see section 5.2.3.3). The exocellular filtered medium, from several points over *A. minutum*'s life cycle, was also assayed for toxicity to *Artemia* in 16h (n=8) brine shrimp bioassays (Appendix 2). The used filters were washed twice with 5ml of 0.65M ammonium formate to remove salts, and stored frozen until used. Once removed from the freezer and thawed, filters were air dried for 24h and toxins extracted from both the cells and filters in 3.0ml of 0.5N acetic acid. The resultant paste (cells, filter and acetic acid) was sonicated (30 s x 3, 50W) in a sonic disintegrator (Tosco) equipped with a rod shaped oscillator. Samples were then refrozen to ensure complete cellular rupture and rethawed, filtered through 0.2µm syringe filters and the pulp discarded. The extract was analysed for GTX toxins using HPLC (Oshima *et al.*, 1988) (Appendix 4). All HPLC analysis was conducted courtesy of A. Negri, Australian Institute of Marine Science, Dampier, Western Australia, with an early contribution by C. Soames, Murdoch University.

5.2.3 Neuroreceptor binding assays (synaptosome binding assays)

Neuroreceptor binding assays are competitive binding assays in which radiolabelled toxin standards compete with unlabelled, unknown toxins for a given number of available receptor sites in a preparation of rat brain synaptosomes. The percentage reduction in radiolabelled toxin binding is directly proportional to the amount of unlabelled toxin present in either a certified reference standard or an unknown sample. Acidic, aqueous shellfish extracts are prepared for testing according to the AOAC (1984) method (Cembella *et al.*, 1995). All neuroreceptor binding assays (STX & PbTx) were conducted courtesy of G. Doucette, Charleston Laboratory, National Marine Fisheries Service, Charleston, USA.

5.2.3.1 Site 1 specific neuroreceptor binding assay

For a complete discussion of the STX neuroreceptor binding assay (i.e. reagents and supplies, preparation of stock solutions and standards, synaptosome preparation) refer Cembella *et al.*, (1995). All cell-free, exocellular samples from cultures 1, 2 & 3 were subjected to site 1 specific, competitive binding assays for SCB toxins.

Assays were carried out in 96 well, polystyrene plates. To each of the 96 wells where added in the following order, 35mL [^{3}H] STX, 35mL STX standard or sample and 135mL synaptosome preparation. The samples were arranged vertically in the plate to simplify organisation after the filters were punched into the vials. The plate was then incubated for one hour at 4°C. The entire plate was then filtered on top of a MultiScreen vacuum manifold. Each well was washed once with 200ml of ice-cold HEPES buffer using a multichannel pipette after which the plastic bottom of the 96-well filter plate was removed and the bottom blotted once on an absorbent towel. The plate was then set into a Multiscreen punch and the disposable punch tips placed on the top of the plate. The wells were punched into the vials which had been pre-filled with about 4ml of Scintiverse liquid scintillant, the caps placed on the vials and each sample vortexed. The vials were then allowed to sit at room temperature overnight and then counted in a standard liquid scintillation counter (results are more easily tabulated if replicates are counted in a consecutive fashion). For sample calculation refer to Cembella *et al.*, (1995).

5.2.3.2 Site 5 specific neuroreceptor binding assay

For a complete discussion of the PbTx neuroreceptor binding assay see Van Dolah *et al.* (1994). Analysis for PbTxs was carried out by binding competition assays with [^{3}H]PbTx-3 for site 5 on the voltage dependent sodium channel in rat brain synaptosomes. Membrane fractions were prepared from the brains of 150-200g Holtzman rats. Brains were homogenized at 4°C in 20mM Tris (pH 7.1) containing 140mM NaCl (12.5ml/brain), incubated at 37°C for one hour, then centrifuged at 54 000g for 15 min. The pellet was resuspended in 20mM Tris/140mM NaCl (10ml/brain) and the protein concentration determined using the micro BCA protein assay. The preparation was aliquoted and stored at -70°C until use.

Assays were carried out in 96-well polystyrene plates in a binding buffer containing 50mM HEPES (pH 7.4), 130mM choline chloride, 5.5mM glucose, 0.8mM magnesium sulphate, 5.4mM potassium chloride, 1mg ml^{-1} BSA, and 0.01% Emulphor-EL 620. Increasing concentrations of [^{3}H]PbTx-3 were added to 140µL of membrane preparation (1mg protein ml^{-1}) to make a total assay volume of 210µL. Incubation were conducted for one hour at 4°C. Non-specific binding was determined by parallel incubations in the presence of a saturating concentration of unlabelled PbTx-3 (10µM), and specific binding calculated by subtracting non-specific binding from total binding at each [^{3}H]PbTx-3 concentration. The membranes were then filtered simultaneously onto a 96 place glass fibre filter mat followed by rinsing the wells four times with ice cold 20mM Tris (pH 7.1). Free [^{3}H]PbTx-3 was determined by directly spotting an aliquote of each concentration of [^{3}H]PbTx-3 onto the filter mat. The mat was dried (60°C) for 15 min then saturated with solid scintillant by heating at 60°C for 5 min. until scintillant was melted and the filter became transparent. The cooled mat was then counted directly in a scintillation counter.

Binding competition assays were also carried out in 96-well polystyrene plates. For generation of PbTx-3 curves, 35µL [^{3}H]PbTx-3 (5nM), 35µL unlabelled PbTx-3 (10^{-6}-10^{-11}M), and 140µL membrane preparation (approx. 1mg ml^{-1} protein concentration) were added to each well. For analysis of unknowns, samples were diluted in 35µL binding buffer. the mixture was then incubated at 4°C for one hour, followed by filtration onto a 96-place filter mat, addition of solid scintillant, and counted as above.

All cell-free, exocellular samples from culture 3 were subjected to the site 5 specific, competitive binding assay for detection of PbTx toxins.

5.2.3.3 Nonspecific, neuroblastoma tissue culture assay

For a complete discussion of the terazolium-based, neuroblastoma tissue culture assay for marine biotoxins active on voltage sensitive sodium channels see Manger *et al.* (1993). This neuroblastoma assay detects biotoxins that interact with voltage-sensitive sodium channels in excitable membranes.The assay is for TTX, STX, PbTx and related toxins based on the combined activities of ouabain (Na^+/K^+ ATPase inhibitor), veratridine (Sodium Channel Activator or SCA) and SCB toxins upon neuroblastoma cells in culture. The mouse neuroblastoma cell line, Neuro 2a, is treated with a fixed concentration of veratridine in the presence of ouabain (to block Na^+ efflux). The combined effect results in elevated intercellular sodium levels leading to altered cell morphology and a subsequent decrease in cell viability. SCBs and related toxins antagonise this effect, maintaining viability in a dose dependent manner. SCAs and related toxins significantly enhance veratridine-induced sodium influx into the neuroblastoma cells thus accelerating the rate of ouabain/veratridine-induced cytotoxicity. Hence although the assay for STXs and NSPs is the same the former measures remaining cellular viability while the latter measures toxin-enhanced cytotoxicity. When both SCBs and SCAs occur together each tends to cancel the other out. A colorimetric reading method is based on the ability of metabolically active cells to reduce a tetrazolium compound, MTT (3-[4, 5-dimethylthiazol-2-yl] -2, 5-diphenyltetrazolium), to a blue coloured formazan product. This method requires only minimal processing and the results can be read on a standard multiwell scanning spectrophotometer (ELISA plate reader).

All neuroblastoma assays for biotoxins active on sodium channels were conducted courtesy of I Garthwaite, New Zealand Pastoral Agriculture Research Institue, Ruakura, New Zealand. The method of Manger (1993) was used with minor modifications (for complete protocol see Garthwaite *et al.*, 1996). Stock cultures of the mouse neuroblastoma cell lines Neuro-2A and NB41A3 were maintained in a humidified incubator (5% CO_2) at 37°C in RPMI medium (RPMI 1640, Gibco BRL 31800-022, New York, NY) supplemented with 10% fetal calf serum, 2mM glutamine, 1mM pyruvate, 100µml penicillin and 100µml streptomycin. Monolayer cultures (5 x 10^4 cells $well^{-1}$) were prepared and incubated for 9-25h with the

sample extract (10µL) or PbTx standard (PbTx-2) in both the presence (test wells) and absence (controls) of ouabain (final assay concentration of 60µM for neuro-2A and 85µM for NB41A3); total test volume was 230µL well^{-1}. Cell viability was determined by aspiration of the medium and incubation of the cell layer with MTT dye solution. A 5mg ml^{-1} stock solution of MTT in 0.01M phosphate buffer (pH 7.4) containing 0.15M NaCL (PBS) was diluted 1:6 with RPMI and 60µL added to each well. The cells were incubated for 20 minutes with the MTT solution, which was removed by aspiration. The precipitated dye was resuspended in 100µL well^{-1} DMSO and absorbance at 595nm determined using a microplate spectrophotometer. A minimum of three replicates of standards and samples were used for each determination of toxin concentration. The assay is regarded as non-specific for NSP toxins (or NSP-like toxins) as it detects activity rather than a specific molecular structure.

5.2.4 Heat stability of exocellular toxic principle

Three, one litre cultures of *A. minutum* where grown as per section 5.2.1. Each culture was set up in conjuction with a control of clean, sterile seawater replete with nutrients (GSe) but remaining non-inoculated as controls. Cultures were grown to the mid/late exponential phase and harvested. Cells were separated from the growth medium via gentle gravity filtration and the cellular fraction was discarded. Each culture and control was divided into 11 equal aliquotes and frozen (-17°C) until use. Once rethawed, each individual aliquot of exocellular medium and a control was subjected to one of 11 different temperatures (17, 20, 30, 40, 50, 60, 65, 70, 80, 90, and 100°C) by immersion in a waterbath and held at that temperature for a period of one hour. The 17°C cell-free culture was not heated but used as a control and kept in a growth cabinet at this normal growing temperature of *A. minutum*. Timing did not commence until the exocellular medium and control had reached the critical temperature. Temperature was monitored ("AquaThermo", -20°C - 70°C ± 0.2°C) both in the waterbath and in the exocellular/control mediums. Once cooled to room temperature, treated medium and corresponding controls were used to challenge *Artemia* in 12hr (n=6) brine shrimp bioassays.

5.3 Results

5.3.1 Endocellular toxicity

Final cell concentrations in cultures 1, 2 & 3, at the end of their growth cycles were high (>10^7

cells L^{-1}). Growth rates of the dinoflagellate cultures varied considerably over the growth cycle. Maximum growth rates achieved were 0.4, 0.7 and 1.1 (K = 1.6, 1.0 and 0.7, respectively) divisions per day for cultures 1, 2 and 3, respectively while peak cell concentrations were 6.59, 10.40 and 17.30 x 10^7 cellsL^{-1}, respectively. Culture growth in carboy 1 (Fig 5.2A) was slower than cultures 2 and 3 (Figs. 5.3A & 5.4A). Peak cell concentrations in culture 1 at the beginning of the stationary phase (day 115-146) where just over half those of culture 2 and just over a third of those in culture 3. An long lag-phase (approximately three months) was observed in all batch cultures of *A. minutum* although this does not occur in small, 250ml cultures and appears to be a phenomenon of large batch cultures of this alga (C. Bolch, pers. comm.) although the initial inoculum was small which may have exacerbated this.

Peak endocellular levels of PSP toxins of 478 and 610 fg STX equiv.$cell^{-1}$ were observed in culture 2 (Fig 5.3B) and 3 (Fig 5.4B) respectively, while in culture 1 (Fig. 5.2B) peak endocellular PSP toxicity only reached 169 fg STX equiv. $cell^{-1}$. Peak endocellular toxicity was reached at the mid/late lag-phase of growth in culture 1 and mid lag-phase in cultures 2 and 3, when cellular concentrations where approximately 2.5 X 10^7 cells.L^{-1}. This concentration corresponded to day 48 in cultures 2 and 3 and day 73, in the slower growing culture 1.

No traces of saxitoxin, neosaxitoxin, sulphamate (GTX_5, GTX_6, C_1, C_2, C_3, & C_4) or decarbamoyl toxins (decarb. saxitoxin, decarb. GTX_2 & decarb. GTX_3) were observed in the endocellular toxic fractions from cells in all cultures. The molar composition of cellular PSP toxin fractions (exclusively Gonyautoxins 1, 2, 3 and 4 for this *A. minutum* isolate) varied considerably over the growth cycle as well as between the three cultures. The slow growing culture 1 had no GTX_4 in the lag phase of growth and only minor percentages of GTX_1. However, in the first assay GTX_1 was close to 45% with an assayed concentration of 36% (Fig. 5.2C). Making up the majority of the toxin during this phase in culture 1 was GTX_2. As the culture entered the exponential phase and through the stationary phase, GTX_2 and GTX_3 became less important, decreasing from 53% GTX_2 and 26% GTX_3 at day 104 down to 18% GTX_2 and 10% GTX_3 at day 168. As a consequence of this GTX_1 increased from 9% (day 104) to 49% (day 168) and GTX_4 increased from 12.6% (day 104) to 23% (day 168).

The within culture variation in the molar percentage toxin profiles of both carboys 2 and 3, in

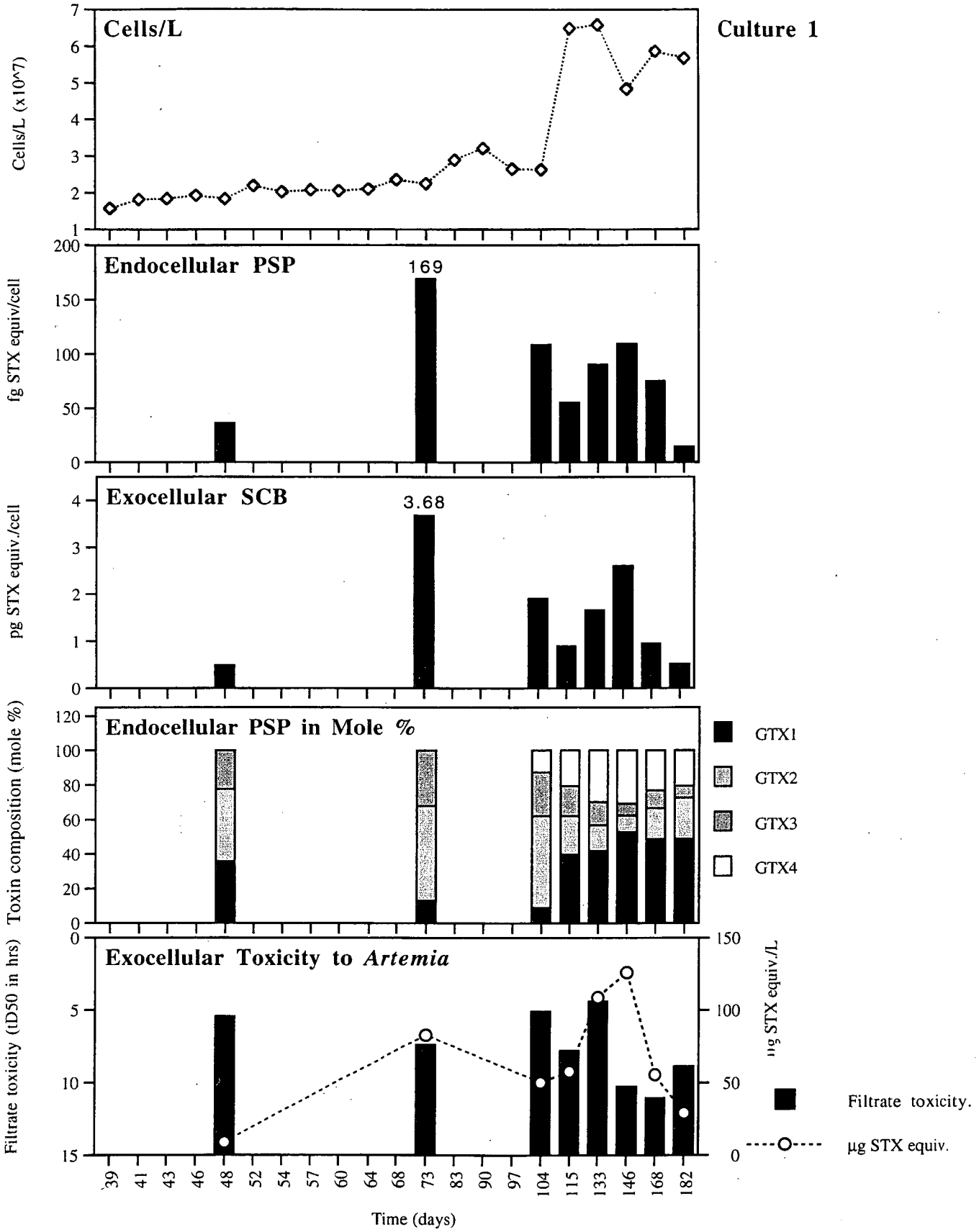

Figure 5.2: Cellular concentration,total endocellular PSP, total exocellular SCB toxins, endocellular % molar fractions of PSPs and toxicity of exocellular medium to *Artemia* in carboy culture 1. Note scale change from endocellular PSPs in femto grams (fg) to exocellular SCBs in pico grams (pg). Note use of reverse scale for exocellular toxicity to *Artemia* (Short tD50 means high toxicity).

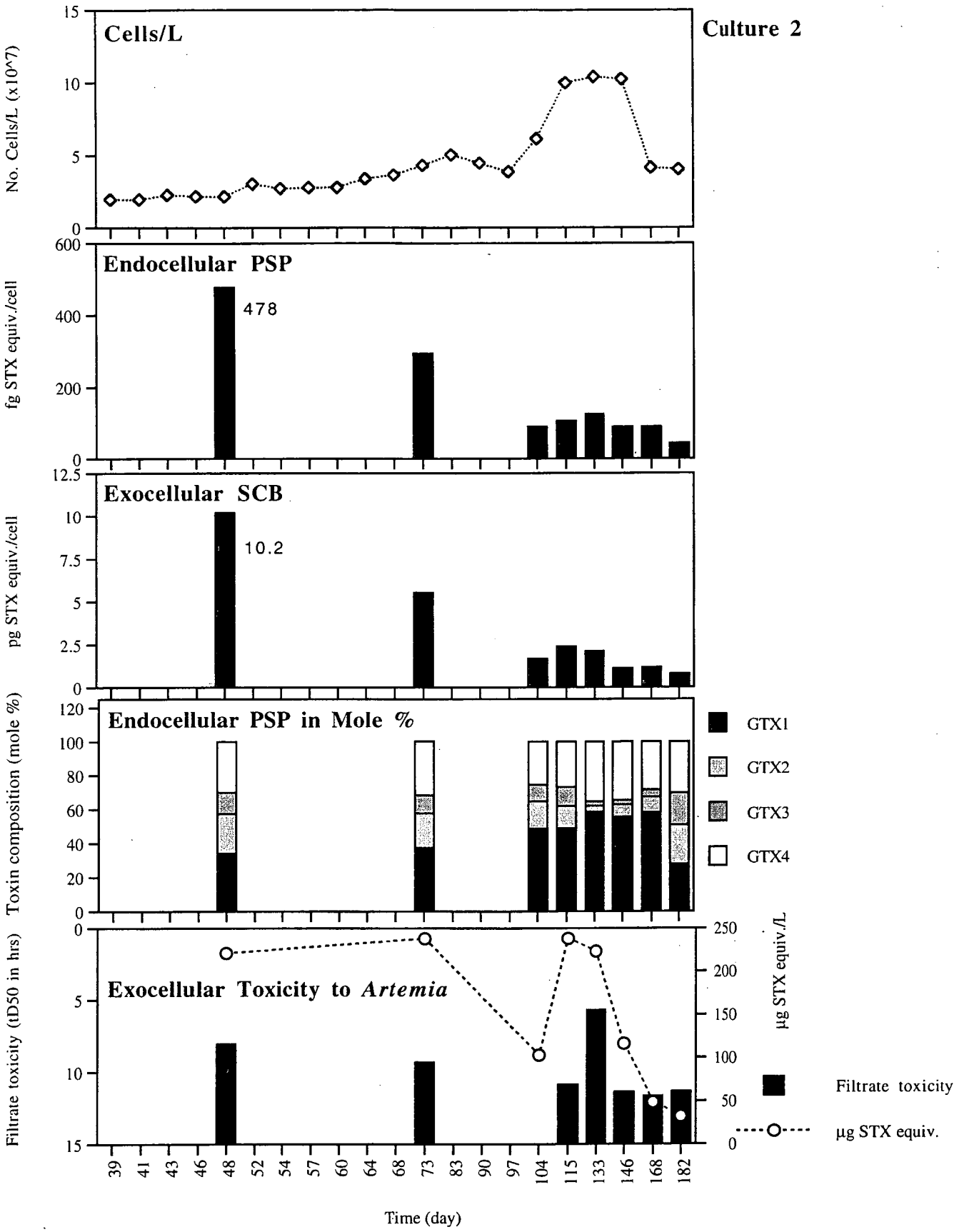

Figure 5.3: Cellular concentration,total endocellular PSP, total exocellular SCB toxins, endocellular % molar fractions of PSPs and toxicity of exocellular medium to *Artemia* in carboy culture 2. Note scale change from endocellular PSPs in femto grams (fg) to exocellular SCBs in pico grams (pg). Make note of reverse scale for exocellular toxicity to *Artemia* (short tD50 means high toxicity).

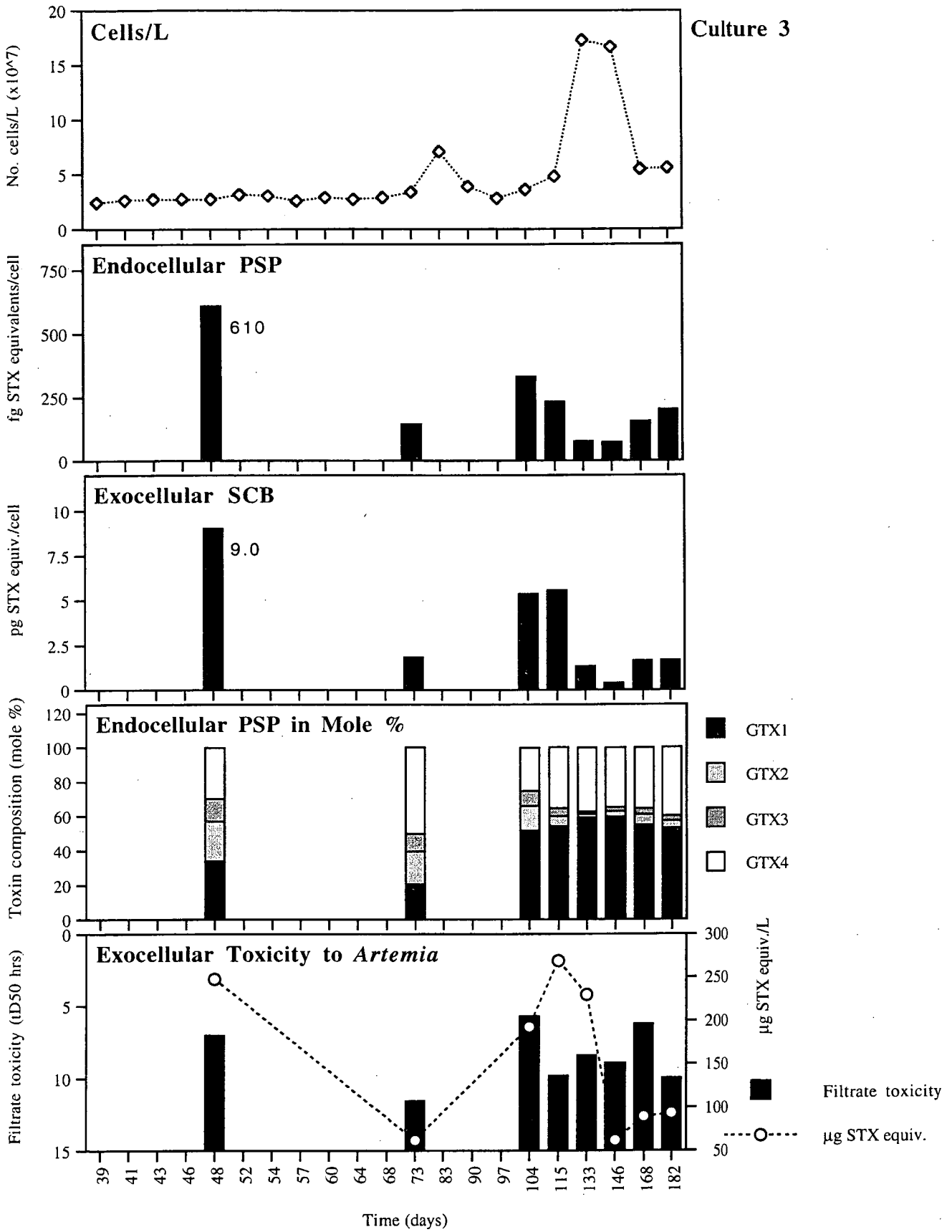

Figure 5.4: Cellular concentration,total endocellular PSP, total exocellular SCB toxins, endocellular % molar fractions of PSPs and toxicity of exocellular medium to *Artemia* in carboy culture 3. Note scale change from endocellular PSPs in femto grams (fg) to exocellular SCBs in pico grams (pg). Make note of reverse scale for exotoxicity to *Artemia* (short tD50 means high toxicity).

terms of variation over the growth cycle, was much reduced (Figs. 5.3D & 5.4D) when compared to culture 1. GTX_4 was present in all phases. In the lag phases the molar percentages of all toxins were relatively similar although there was a spike of GTX_4 (50.3%) in culture 3 at day 73. GTX_2 and GTX_3 were relatively important contributors but, as both cultures moved into exponential and stationary phases, GTX_1 and GTX_4 increased in importance. In nearly all cases (except day 182 in culture 2 and day 73 in culture 3) the molar percentage contributions of toxins in cultures 2 and 3 where in order of decreasing importance $GTX_1 > GTX_4 > GTX_2 > GTX_3$.

5.3.2 Exocellular toxicity

The time course of exocellular SCB toxicity per cell in the *A. minutum* cultures (Figs. 5.2C, 5.3C & 5.4C) appeared to follow that of endocellular PSP toxicity but at a concentration per cell three orders of magnitude higher (10^{-12}) than assayed for the endocellular toxic fraction per cell (10^{-15}). Again, in culture 1 (Fig. 5.2C) the peak exocellular concentration occurred in the mid/late-lag phase on day 73 while in cultures 2 (Fig. 5.3C) and 3 (Fig. 5.4C) the peak, exocellular SCB toxin concentration occurred in mid-lag phase on day 48. Generally, as the cultures aged the activity of SCB substances declined with this decline continuing into the death phase.

After the SCB assays were carried out, samples where stored frozen for one year and re-assayed. A subsequent drop in SCB activity (20-25%) appeared to have occurred over this time period indicating SCB toxin degradation (G. Doucette, pers. comm.).

The toxicity of the cell free culture medium from cultures 1, 2 & 3 to *Artemia*, was compared with the same medium's (exocellular) SCB activity expressed in STX equiv. L^{-1} (Figs. 5.2E, 5.3E & 5.4E). Dinoflagellate-free culture media was always toxic to 4-day old *Artemia* resulting in tD50 values ranging from 4.5 to 12 hours. Peak culture-filtrate toxicities occurred at day 133 (mid stationary phase) in cultures 1 and 2 but earlier in culture 3 on day 104 (early exponential phase). No toxic response was observed in the controls. The toxicity of the culture-filtrate did not appear to correlate with the concentration of SCB substances in the exocellular medium.

Neuroblastoma bioassay also confirmed the presence of SCB substances in the culture-filtrate (Table 5.3) of *A. minutum*. However, the presence of another, unknown substance in the medium that had SCA activity confounded the SCB results.

A high degree of SCA activity was found in the culture-filtrate of *A. minutum*, particularly in cultures 2 and 3 (Table 5.3). The tetrazolium based neuroblastoma assay does not clarify the identity of a substance beyond the fact that it had an antagonistic effect to the SCB substances present. Brevetoxins are powerful SCA toxins and therefore their presence in the exocellular medium of *A. minutum* was examined using a competitive binding, neuroreceptor assay relying on competition for site 5 on the voltage dependent sodium channel. No brevetoxins where found in any of the time course samples from culture 3. Funds were not available for a similar examination of cultures 2 and 3 for PbTxs.

Table 5.3: Activity of sodium channel blocking substances (STX equiv.) and sodium channel activating substances in exocellular medium of *A. minutum* assayed by neuroblastoma, tissue culture bioassay.

	Day							
	48	73	104	115	133	146	168	182
Culture 1								
SCB (ng/ml)	5	25	20	<5	<5	<5	-	-
SCA	-	-	-	-	-	-	++++	+++
Culture 2								
SCB (ng/ml)	-	-	-	-	-	-	-	-
SCA	-	-	++++	+++	++++	++++	++++	++++
Culture 3								
SCB (ng/ml)	10	10	-	15	-	-	-	-
SCA	-	-	++	-	+++	++++	++++	++++

Note: SCB = sodium channel blocking substances

SCA = sodium channel activating substances (++++ > +++ > ++ > -)

5.3.3 Heat stability of exocellular toxic principle

Heat stability of the exocellular toxic principle did not seem to be uniformly affected by temperature (Fig. 5.5). An *Artemia* bioassay using control, culture-filtrate that had been held at 17°C set a normal exocellular tD50 toxicity level of 2.27 ± 0.62h. A significant drop in toxicity

(tD50) did not appear to occur until the exocellular medium was heated to 90°C, while a significant *increase* in toxicity was observed after the medium had been subjected to temperatures of 50°C and 60°C. A drop in toxicity was observed at 65°C, although this was small with the overall toxicity still remaining high (approximately 4-5h tD50). This drop in toxicity was also witnessed after the 40°C treatment however toxicity again rose when the exocellular medium was heated to 50°C. Another significant drop in toxicity was observed after the 90°C and 100°C treatments where toxicity dropped to 12 and 9h respectively. This possibly suggests more than one toxic principle with one being "denatured" at 30-40°C and the second at >80°C. Control media appeared unaffected by heat treatments and were non-toxic to *Artemia* in all exposure cases.

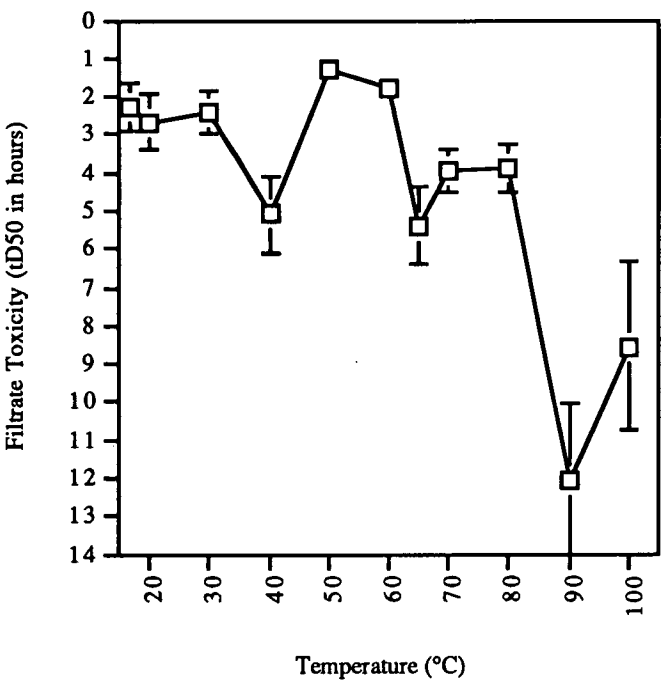

Figure 5.5: Time to death of 50% of *Artemia* (tD50's) exposed to cell-free *A. minutum* growth medium after heat treatment. Note use of reverse scale (short tD50 = high toxicity).

5.4 Discussion

5.4.1 Timing of peak toxicity

A positive correlation was observed between the time course of endocellular and exocellular

SCB toxins in *A. minutum* cultures. These observations suggest that the endocellular PSP toxins may be linked to the SCBs in the exocellular medium, although no confirmation of the identity of the exocellular SCBs is possible at present, although Hsieh *et al.* (2000 in press) recently found high concentrations of C-toxins in the extracellular culture medium of *A. tamarense*. Cellular densities reached were very high ($>10^7$ cells L^{-1}) however, not as high as those reported in the literature by Cannon (1990) for wild blooms of *A. minutum* occurring in the Port River, Adelaide. A discrepancy occurred between the timing of the peak in cellular toxicity in the strain of *A. minutum* used in this work and others reported in the literature. Maximal, total (endocellular & exocellular) toxicity was reached in the lag phase of growth, before the exponential phase and this peak toxicity began to decline as the culture entered exponential and stationary phases. This contradicts observations by Kim *et al.* (1993) who found peak toxicity in *A. minutum* and other members of the genus *Alexandrium* at very early to mid-exponential phase (Mascarenhas *et al.*, 1995). Conversly, the toxicity of *A. catenella* was highest in the lag phase but decreased when cells entered the exponential and stationary phases (Sakamoto *et al.*, 1992). Soames (pers. comm.,1995) found that peak toxicity was reached at early exponential phase in batch cultures of *A. minutum* while Anderson *et al.* (1990) reported peak toxicity more towards the mid-exponential phase in *A. fundyense* and *A. tamarense*. Similarly, Kim *et al.* (1993) found a correlation between increased cell toxicity and early exponential growth in *A. minutum* while Boczar *et al.* (1988) showed that the highest STX concentrations of *A. tamarense* occurred at the mid-exponential growth phase. Total SCB toxicity was generally the lowest in the stationary and death phases.

5.4.2 Components of toxicity in mole %

If the peaks in endocellular and exocellular toxicity are analysed according to the molar percentage of the component toxins it is apparent that the peak levels of toxicity occur concurrent with the highest production of the epimeric pair GTX_2/GTX_3 (Table 5.4). GTX_3 is the most potent of all the PSP toxic fractions having a higher toxic potency than STX (see Oshima *et al.*, 1989). In culture 1, peak toxicity was reached at day 73 when 87% of the total toxin production (see Figs 5.2B & C) was contributed by GTX_2/GTX_3. Although peak toxicity was reached earlier in culture 3 (day 48), again this corresponded to the peak in GTX_2 and GTX_3 production, (the much lower peak toxicity found in culture 1 compared to culture 3 [see Figs 5.2B & C compared to Figs 5.4B & C respectively] is more than likely due to the lower

total cellular density in culture 1 on day 73 [2.25 x 10^7 cells L^{-1}] when compared to the total cellular density in culture 3 at the time of it's peak in toxicity on day 48 [2.75 x 10^7 cells L^{-1}]). Peak total toxicity in culture 2 occurred on day 48 (see Figs 5.3B & C) as it did in culture 1. However, the highest contribution of GTX_2/GTX_3 occurred on day 182 (death phase) when this pair of toxins contributed 41.9% of the total molar toxin percentage. The molar percentage contribution of GTX_2/GTX_3 on day 48 was not greatly dissimilar to this with GTX_2 contributing only 0.1% less than on day 182 whereas GTX_3 contributed 6% less (see Table 5.4), a total combined molar percentage contribution of 35.8%.

Table 5.4: Highest proportions (mole %) of toxic fractions and days post inoculation when they occur.

Gonyautoxin	Culture 1		Culture 2		Culture 3	
(GTX)	day	mole %	day	mole %	day	mole %
1	146	52.6	133	58.5	146	59.6
2	73	54.8	48/182	23.1/23.2	48	23.1
3	73	32.2	48/182	12.7/18.7	48	13.2
4	146	31	133	35.4	73	50.3

Hallegraeff *et al.* (1991) reported the mole percentage of endocellular GTX toxins in *A. minutum* to be: GTX_1 = 45.2%; GTX_2 = 15.7%; GTX_3 = 10.8%; GTX_4 = 27.5%. It would be expected that this molar ratio of endocellular toxins would be stable throughout the growth cycle of the batch culture even though the concentrations of individual toxic components may vary. However, in the present work there was wide variation in the molar percentage of GTX toxins between cultures over the growth cycle. This supports work by Anderson (1990) who describes highly variable toxicity in the genus *Alexandrium* both between isolates of the same species and single isolates. Cembella (1998) and Oshima *et al.* (1993) suggest that the high ratio (often >10:1) of ß:å 11-hydroxysulfated derivatives found in *Alexandrium* sp. and *Gymnodinium catenatum* after careful extraction of the cells indicates that only the 11-ß-epimers (C_2, C_4, GTX_3, GTX_4) are biosynthesised and that the 11-å-epimers (C_1, C_3, GTX_1, GTX_2) are formed *in vitro* by spontaneous epimerization. This was contradicted by the present work in which in all cultures endocellular toxicity, particularly in early culture growth, was dominated by GTX_1 and GTX_2. No GTX_4 was detected at all in the lag phase of culture 1 while in the same period in both cultures 2 and 3, GTX_1 and GTX_2 were the major toxic components of the endocellular toxicity. In all cases however, GTX_4 became more important as

the culture aged as did GTX_1 with the epimeric pair GTX_2/GTX_3 declining in importance until the toxin composition resembled that of the *A. minutum* described by Hallegraeff *et al.* (1991). This may suggest that the SCBs in dinoflagellates are a chemical defence system with the fraction of highest potency (GTX_3 with its epimer GTX_2) being produced as the culture ages. It is unfortunate that currently available analytical methodologies are unable to resolve exocellular SCB toxicity into its constituent components.

5.4.3 The Role of SCB and SCA exocellular toxicity

Three orders of magnitude disparity was observed between the concentration of SCBs within individual dinoflagellate cells (fg) and the concentration of SCBs in the exocellular medium (pg) per cell. This observation indicates that in the midst of a bloom of *A. minutum* the majority of SCB toxins exist in the exocellular medium or the water column, rather than inside the cell as previously thought. This is what one would expect of a substance whose primary role was allelopathic or defensive. Biosynthetic production of the SCBs clearly is inside the cell however the target for their role is outside the cell. Compared to the endocellular environment which has a finite volume, the exocellular environment is infinite potentially explaining the orders of magnitude higher SCB concentrations. What is puzzling however, is the presence of another substance acting not unlike NSP toxins (but not PbTx) and which has not previously been reported from any species of *Alexandrium*. The tetrazolium based, neuroblastoma tissue culture bioassay is a non-specific assay that reacts to substances that bind to both site 1 (SCB or STX-like) and site 5 (SCA or PbTx-like) on the sodium channels of neuroblastoma cells, hence the presence of both types of substances interfere in accurate measurement of either one alone. Where concentration values of SCBs are given these appear to be overestimations of the values reported using the competitive binding, neuroreceptor assay of Doucette *et al.* which is specific for toxins binding to site 1 on the voltage dependent sodium channel, such as PSP toxins and other SCBs. This competitive binding type assay is not affected by substances that bind to the sodium channel at other sites (G. Doucette: pers comm.).

5.4.4 Reduction in exocellular toxicity with heating

A significant reduction in the toxicity of *A. minutum* culture-filtrate to *Artemia* was observed when heated above an 80°C threshold. This tends to indicate that the exocellular toxic

principle(s) is heat labile to some degree. Although the basic PSP toxin structure is heat stable (Genenah and Shimizu 1981, Stanley and Brown 1988, Laycock *et al.* 1995) interconversions between many if not all of the PSP toxin derivatives are achievable by manipulation of conditions, particularly temperature and pH (Laycock *et al.* 1995). The consequence of such interconversions are small in terms of changes in the overall structure of the PSP toxin molecule but large in terms of overall toxicity. For example, a large proportion of the PSP toxins that accumulate in dinoflagellates are the relatively low toxicity C-fraction toxins that contain two sulfate groups. These groups can be easily removed producing the vastly more potent GTX toxins that were not abundant in the algal source (Laycock *et al.* 1995). Hence, even though Ghazarossian *et al.*(1976, in: Schantz 1986) found that the removal of the carbamoyl group at C-13 in STX after treatment with 7N HCl at 100°C reduced the STX toxicity to about 60% most recent data indicates that upon heating (100°C) and weak acid hydrolysis (of the R4 group, see Fig. 1.1) toxicity of some PSP toxin fractions *increases*. Under such conditions weaker N-sulfocarbamoyl PSP toxins can be converted to the much more potent carbamate toxins with a concomitant 10-100 fold increase in toxicity (Hall and Reichardt, 1984, Carmichael, 1986, Oshima *et al.*, 1987, Hallegraeff 1989). Similarly, Oshima *et al.* (1987) found high yields of decarbamoyl-GTXs 1-4 when C-fraction toxins were heated (cooked). The reduction in toxicity above 80°C observed here reinforces the suggestion that the toxic principle(s) in the exocellular medium is not a PSP toxin.

A significant reduction in toxicity of the culture filtrate to *Artemia* was also found after the culture filtrate was heated to 30°C. It could be proposed that such an observation indicates several "toxic principles" with the effects of these "toxic principles" being observed not in isolation but in concert. 30°C may be the upper thermal limit for one principle, observed as an immediate decline in toxicity, but the lower limit for another that becomes more toxic as temperature increases observed as a gradual increase in toxicity after 40°C. This does support the notion that the culture filtrate of *A. minutum* contains more than one toxic principle.

5.4.5 Conclusion

The aetiology of the toxicity of the *A. minutum* medium to *Artemia* has yet to be clarified. Pure PSP toxins in solution do not kill *Artemia* (see section 4.3.3.2.2, [Trial E]). However algae that produce SCA toxins (such as NSPs) do kill *Artemia* and also cause pathological

tissue damage in fish (Endo *et al.* 1985). The death of *Artemia* (presented as tD50's) from *A. minutum*'s cell-free growth medium appeared to be completely independent from the concentrations of both endocellular and exocellular SCB toxins and composition of the endocellular toxins. Similarly, there appeared to be no correlation between growth stage and the highest toxicity of the medium towards *Artemia* although this peak in toxicity was always in the exponential/stationary phase when cellular density was at its peak. This was also the case with the concentration of exocellular SCB substances on a per cell basis (see Figs. 5.2C, 5.3C & 5.4C) when compared to cellular density (see Figs. 5.2A, 5.3A & 5.4A). Similarly, although the toxicity of some PSP toxins is affected by heat, the effect is normally to *increase* toxicity and not the decrease in toxicity that was observed here. These observations strongly suggest, together with the data from Chapter 4, that another unknown toxic principle(s) is active in the exocellular medium of *A. minutum*.

Chapter 6: The role of bacteria in the exotoxicity of *Alexandrium minutum*

6.1 Introduction

The role of bacteria in algal-PSP toxicity is vigorously debated in the literature. This dates back largely to the claim by Kodama (Kodama *et al.*, 1988) who isolated an STX producing endobacterium from *Alexandrium tamarense* (reported as *Protogonyaulax tamarensis*). Since that time many researchers have reported on bacterial production of STXs (Kodama *et al.*, 1993, Levasseur *et al.*, 1996, Shimizu *et al.*, 1996, Doucette *et al.*, 1998) from species such as *Moraxella* sp, *Pseudomonas stutzeri*, *P. diminuta*, *Bacillus* sp, *Alteromonas* sp. and *Vibrio* sp.. Furthermore many dinoflagellate species have been observed to have associations with PSP toxin producing bacteria (e.g. *A. tamarense* , *A. minutum*, *A. cohorticula*, *A. catenella* and *Gymnodinium catenatum*) (Kodama *et al.*, 1989, Kodama *et al.*, 1990, Rausch de Traubenberg & Lassus, 1991, Doucette & Trick, 1995, Franca *et al.*, 1995, Franca *et al.*, 1996, Shimizu *et al.*, 1996) with these bacteria being either endocellular, exocellular or both.

The close spatial and temporal coupling of bacterial and algal populations in the ocean is well documented as is the tendency of both groups to synthesize metabolites that may be beneficial or harmful to one another (Kjelleberg *et al.*, 1993). The presence of bacteria in the water column, attached to the outer surface (Kogure *et al.*, 1982) and more importantly the inner cell surface, of dinoflagellates, underneath the amphiesma and in the cytoplasm (Franca *et al.*, 1995, Mascarenhas *et al.*, 1995) introduces complications to experimentation attempting to investigate autonomous dinoflagellate toxicity. Whereas it is possible to rid dinoflagellate cultures of bacteria outside the algal cells or attached to them, it is impossible to achieve axenicity in the dinoflagellate cytoplasm down to the molecular level (i.e. containing no intact, actively transcribed bacterial genome wether or not it is contained within a recognizable bacterial cell) (Doucette *et al.*, 1998).

Although the production of PSP toxins by bacterial cells has been shown to be small when compared to that of dinoflagellate cells, typically 5% for *Moraxella* sp when compared to the dinoflagellate *Alexandrium* (Doucette & Trick, 1995), intoxication of higher trophic organisms, such as bivalves, has been observed in the absence of toxic dinoflagellates but in

the presence of bacterial species that produce STXs autonomously. For example, Sakamoto *et al.* (Sakamoto *et al.*, 1992) observed PSP toxicity in bivalves in Ofunato Bay (Japan) in 1980 and 1986 to be out of phase with blooms of the dinoflagellate *A. catenella*. The dinoflagellate cells themselves had very low PSP toxicity while the 5-20μm particulate fraction (smaller than *A. catenella*) of the water column showed high toxicity. Similarly, Kodama (1989) and Kodama *et al.* (1990) reported on the apparent toxicity of scallops (*Patinopecten yessoensis*) in the same area in the absence of *A. tamarense* (reported as *P. tamarensis*). Peak toxicity was found in particulate matter from the water column in the size range encompassing bacteria of 0.45 - 5.0μm. Toxicity was not found in any other size fraction with the seawater and cultured medium of *A. tamarense* reacted positively with anti-*Moraxella* sp. serum for the presence of this bacterium.

Maas (1997) demonstrated that reducing the bacterial populations associated with *A. minutum* reduced endocellular toxin production by 50%, and could be increased again by restoring the bacteria. Ogata *et al.* (1990) indicate that toxic dinoflagellates are always contaminated by toxic bacteria while no bacteria are *isolated* from non-toxic strains of dinoflagellates (although they are probably still present), whereas Tosteson *et al.* (1989) confounded the picture by indicating a loss of toxicity in *Gymnodinium veneficum* Ballantine when cultured under axenic conditions but no loss of toxicity in *G. breve* (Davis) (brevetoxins) when cultured in the same manner. Furthermore, bacterial extracts of *Gambierdiscus toxicus* and *Ostreopsis lenticularis* where found to be non-toxic but dinoflagellate toxicity was related to the number of bacteria directly associated with the algal cells. The transfer of toxicity from toxic to hitherto non-toxic dinoflagellates, using toxic bacteria as the vector, has also been claimed. Silva & Sousa (1981) claimed transfer of toxicity to a non-toxic *Gyrodinium* strain using a *Pseudomonas* sp. isolated from a toxic *A. tamarense* (reported as *P. tamarensis*) strain, while non-toxic clones of *Prorocentrum minimum*, *Gyrodinium instriatum* and *A. tamarense* became toxic after inoculation with a bacterial strain isolated from a toxic strain of *A. tamarense* (Rausch de Traubenberg & Lassus, 1991). This could not be confirmed by Dimanlig & Taylor (1985) who were unable to cross-transfer toxic ability between toxic and non-toxic dinoflagellate strains using exocellular bacteria.

Although bacterial production of PSP toxins has been claimed and a role for these PSP toxin producing bacteria in algal toxicity can be hypothesised, data on bacteria producing other forms

of algal toxins (ciguatoxins, DSP, brevetoxins, yessotoxins, NSP, hemolysins, cytotoxins) is poor or non-existent. Douglas & Bates (1992) have reported on axenic cultures of *Pseudonitzschia multiseries* that produce domoic acid but at a level 20 times less than their xenic counterparts (Douglas *et al.*, 1993). Reintroduction of bacteria to the same axenic cultures resulted in an enhancement of domoic acid production by 2-115 fold, depending on the *P. multiseries* and bacterial strain used (Bates, 1998). Currently, the "bacterial hypothesis" does not extend to exotoxic substances, such as those responsible for hemolytic ichthyotoxicity (Rausch de Traubenberg & Lassus, 1991).

The following chapter attempts to determine whether bacteria, inherent in the cultures of *A. minutum* (strain AMAD-06), are contributing to the demonstrated toxicity of the dinoflagellate growth medium described in the previous chapters. *Artemia* bioassays were used as described earlier but after reductions in bacterial loads within the dinoflagellate cultures using antibiotics.

6.2 Materials and Methods

6.2.1 Algal growth and bacterial inhibition via continuous antibiotic exposure

The antibiotic resistance of *A. minutum* and possible contaminating bacteria were tested by adding 10, 25, 50, 100, 250, 500 and 1000μgml^{-1} of each antibiotic (penicillin G, dihydrostreptomycin, neomycin and amphotericin)(Divan & Schnoes, 1982) to triplicate cultures. Individual, 125ml erlenmeyer flasks (78 in total) were filled with 75ml of clean sterile seawater containing GSe nutrients (Appendix 1). A control was also set up containing no antibiotics. Each flask was inoculated with 1.0ml of actively growing *A. minutum* culture. Cultures were left to grow (12hr light/12hr dark, 17°C) for 20 days after which time a 4.0 ml subsample was taken. Glutaraldehyde was added to the subsamples (final concentration of 2%) and the number of algal cells in each subsample was counted.

6.2.2 Algal growth and bacterial inhibition via intermittent antibiotic exposure

A 1.0ml inoculum of actively growing *A. minutum* was placed in five wells of two sterile, 12

well microplates (IWAKI, polystyrene 3815-012). To these wells was added 4.0ml of sterile GSe medium containing penicillin (final conc., 50μgml^{-1}) (Fig. 6.1). The algae were exposed to the penicillin for 24h. After this time 1.0ml of culture was withdrawn from each well and placed in individual, 20ml erlenmeyer flasks containing 9.0ml of sterile GSe medium. The alga was left to recover in these flasks for 10 days. As this initial recovery would be in the presence of residual antibiotic, a second and third transfer was included to successively reduce the amount of antibiotic present. In this way, antibiotics present in the medium where successively diluted by a factor of ten after each recovery transfer. This method of exposure and recovery appeared to cause the least disruption to growth with the maximum bactericidal affect.

After the third recovery period, a 1.0ml inoculum was taken from each flask and exposed to the second antibiotic (neomycin, final conc. 500μgml^{-1}), transferred through the three recovery phases and so on through the third antibiotic (dihydrostreptomycin, final conc. 500μgml^{-1}) exposure and the fourth antibiotic-combination exposure (final concentrations were penicillin, 50μgml^{-1}; neomycin, 500μgml^{-1}; dihydrostreptomycin, 500μgml^{-1}). After each recovery growout culture medium was removed with a sterile wire, streaked onto agar (SWAV; see Appendix 5) and left for 3-4 days (12hr light/12hr dark, 17°C) as a qualitative test for bacterial presence This was coupled with a quantitative count under the fluorescence microscope but only if no bacterial growth was observed on the agar plate. After the final exposure to antibiotics and the recovery period all cultures were allowed to grow to mid exponential phase and then were checked for bacteria by fluorescence microscopy (Zeiss Axiovert 35) (Fig. 6.1).

Once the number of bacterial cells in each culture was counted, the cultures were filtered through GF/F (Whatman) filters. A 2.0 ml aliquot of the filtrate was taken and analysed for SCB toxin content using the neuroblastoma tissue culture assay assay of Garthwaite *et al.* (see Chapter 4), the remaining filtrate was used to challenge *Artemia* in triplicate, 26 h (n = 13) brine shrimp bioassays.

6.2.3 Bacterial cell counting

Preparation of all materials and slides for bacterial counting were carried out in a sterile laminar flow cabinet. All equipment and glassware had previously been sterilized by autoclaving and were constantly washed with sterile, deionized water to remove any particulate matter.

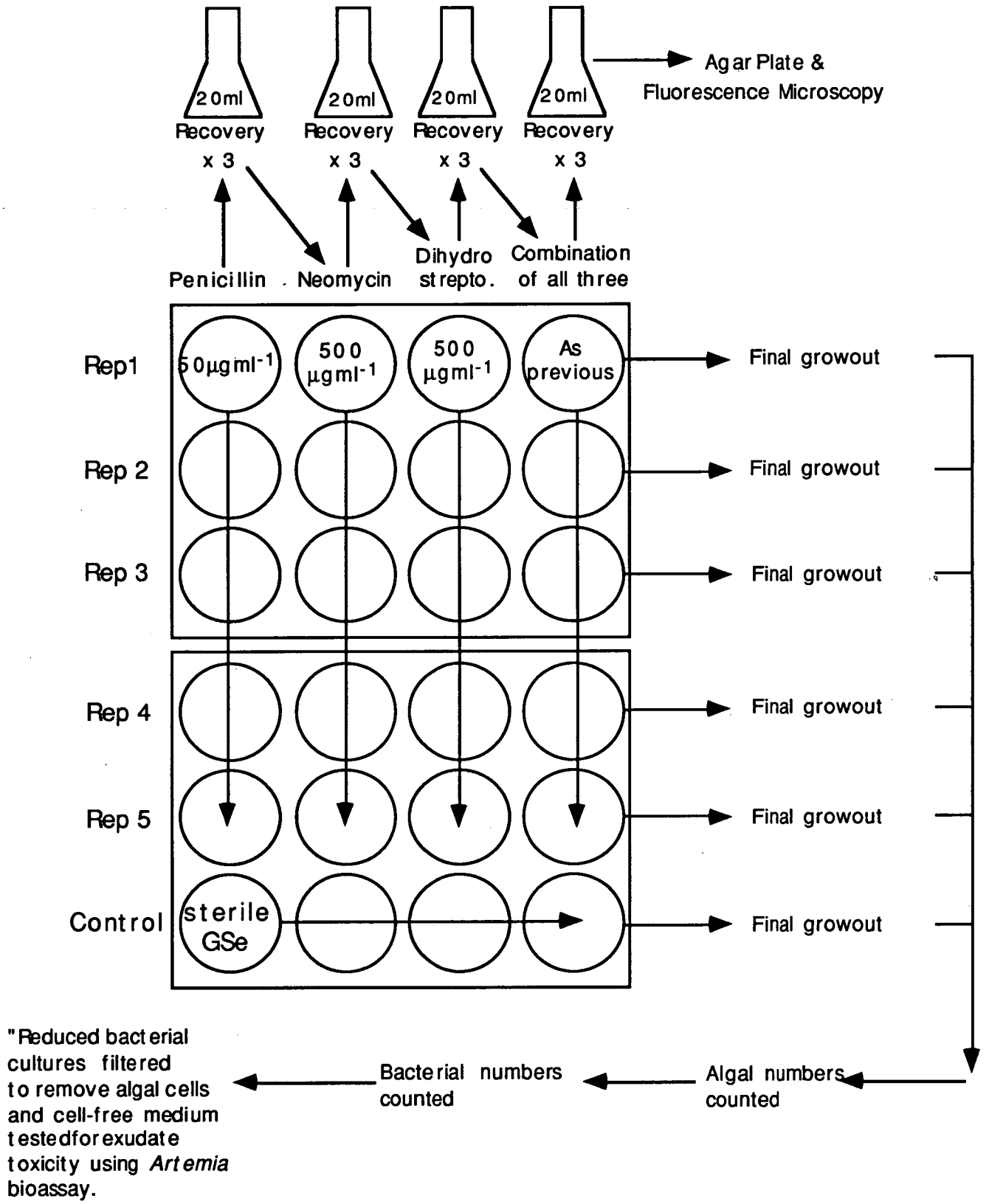

Figure 6.1: Schematic diagram indicating the growth of *A. minutum* cultures intermittant antibiotic exposure and recovery periods.

Cultures were preserved with 1-2% glutaraldehyde and stored in the dark at 4°C until used. The bacteria were stained with 4", 6" - diamidino-2-phenylindole (DAPI) (Sigma) for approximately 30 minutes, filtered onto black 0.22µm-pore-size membrane filters, mounted onto slides sealed with clear nail polish and cells counted with a Leitz fluorescent microscope at a magnification of 1000x, within 3 days (see: Lovejoy *et al.*, 1998). Bacterial cells fluoresced blue and counting was based on 20 random fields of view. Slides were replicated three times.

6.3 Results

6.3.1 Continuous antibiotic exposure

Cultures of *A. minutum* did not respond well to continuous exposure to antibiotics. Culture growth was retarded with cellular densities remaining at a level ($<5.0 \times 10^3$ cells ml^{-1}) corresponding with the lag phase in control cultures which reached cell concentrations in excess of 8.0×10^4 cells ml^{-1}. Cellular morphology was abnormal with cells being pale in colour and slightly elongated (33µm) along the anterior/posterior axis, giving the cells a more pointed than spherical resemblance. Cells remained clumped at the bottom of the flasks and not near the surface of the culture medium as is usually the case for this isolate. Cultures remained at low cellular densities and never grew even after 20 days. Due to the adverse reaction of the cultures to the continuous exposure to antibiotics no further data was collected on these cultures including bacterial counting. Continuous antibiotic exposure was abandoned as a method of reducing bacterial load in *A. minutum* cultures.

6.3.2 Intermittent antibiotic exposure

A. minutum cultures seemed to respond more favourably to exposure with antibiotics followed by fixed periods of recovery. The cultures seemed to grow well in the recovery phases although slightly slower than normal. Morphology of the *A. minutum* cells was normal. Bacterial loads in each of the antibiotic treated cultures were significantly reduced (>80%) by the end of the full spectrum of exposures when compared to the untreated control. However, total axenicity was not achieved possibly indicating a very close algal/bacterial relationship as proposed by Nayak *et al.* (1997) for *Amphidinium carterae*.

Penicillin seemed the least efficient at reducing bacterial load with all cultures showing rapid growth of bacterial colonies in the agar test (Table 6.1). In all cases where bacteria grew on SWAV plates the colonies were small and round with a shiny milk-yellow colour. Neomycin exhibited some ability in reducing bacteria within the cultures, however once this antibiotic became significantly diluted in the growout phases, the bacteria seemed to easily re-establish themselves shown by colony presence in the second and third agar tests.

Table 6.1: Presence (+) or absence (-) of bacterial growth on agar plates (SWAV) post antibiotic treatment and recovery phases. Also indicated are percentage reduction in bacterial loads in post final growout (n = 20), the effects of the treated and control culture-filtrate on 4-day old *Artemia* (tD50 in hours) (n = 13), and SCB content of filtrate in STX equivalents.

	Bacterial Growth					
Antibiotic	**1**	**2**	**3**	**4**	**5**	**Control**
Penicillin	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+
Neomycin	-/+/+	-/-/+	-/+/+	-/+/+	+/+/+	+/+/+
dihydrostrepto.	-/-/+	-/-/-	-/+/+	-/-/+	-/-/-	+/+/+
combination	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	+/+/+
	Results					
final bacterial load (10000 x cells/ml)	0.48 ±0.04	0.30 ±0.02	0.37 ±0.03	0.52 ±0.02	0.82 ±0.04	4.47 ±0.15
reduction of bacteria (%)	89	93	92	88	82	0
tD50 (hrs) of *Artemia*	22.9 ±6.1	21.9 ±5.5	21.1 ±5.5	18.4 ±4.2	16.1 ±3.1	12.2 ±2.1
SCB activity (ng STX equiv./ml)	0	10	20	20	20	20

Dihydrostreptomycin and the combined efficacy of all three antibiotics in the third and fourth treatments appeared to be the most effective at reducing bacterial loads to a point where they did not appear on the three successive agar tests which prompted the work to progress to the second stage of bacterial enumeration via fluorescence microscopy and *Artemia* bioassays. The control culture, which was not exposed to any antibiotics had the highest bacterial load (Table 6.1) at the end of the experimental period. It also had the highest toxicity towards 4-day old *Artemia* with a tD50 of 12.2 ± 2.1 hours (Table 6.1). In the treated cultures reductions in

bacterial loads appeared to have a positive correlation with culture-filtrate toxicity (Table 6.1) in the toxicity sequence: control > replicate 5 (82%) > replicate 4 (88%) > replicate 3 (92%) > replicate 2 (93%). Only replicate 1 did not fit this sequence having an 89% reduction in bacterial load, however having the lowest of all treatment toxicities towards *Artemia*. Also, low toxicity of antibiotically treated culture-filtrate of *A. minutum* towards *Artemia* seemed to correspond to a higher degree of variability, the lowest variability being in the untreated control, although the control had the highest toxicity, with the opposite in rep. 1 which exhibited the lowest toxicity however the highest variability.

Toxicity of the exocellular medium to *Artemia* did not appear to be linked to SCB concentration. The least toxic (to *Artemia*) replicates (1 and 2 see Table 6.1) had the lowest measurable levels of SCB (0 and 10ng STX equiv. ml^{-1} respectively) however, the exocellular medium of all other replicates, including the control, appeared to contain the same concentration of SCBs (20ng STX equiv. ml^{-1}).

6.4 Discussion

Bacterial interactions with harmful algal bloom species are increasingly cited as potentially important regulators of algal growth and importantly, toxicity (Rausch de Traubenberg & Lassus, 1991, Doucette *et al.*, 1998). The question now does not appear to be *whether* bacterial/algal interactions play a role but *how* they are manifested and by *what* mechanisms. It has been well documented that bacteria aid in the promotion of algal growth (Maruyama *et al.*, 1986, Nishijima & Hata, 1989) usually by the production of excreted metabolites (Sawayama *et al.*, 1993, Doucette, 1995) and are involved in initiation and maintenance of stationary growth phases of algal blooms (Smayda, 1996) and cell death via broad spectrum algicides (Doucette *et al.*, 1998). Influence of bacteria over algal cell death may play a role in driving the phytoplankton assemblage towards dominance by a different algal taxon and cause the collapse of algal blooms. Similarly, species of algae have been found to produce bactericides that exert some control over closely associated bacteria (Nelinda *et al.*, 1985, Kellam & Walker, 1989, Gromov *et al.*, 1991). Such capabilities imply a potential for feedback mechanisms which may ultimately play an important role in controlling both bacterial and algal species succession and population dynamics (Doucette *et al.*, 1998).

Many marine bacteria associated with microalgae have been found to be able to produce Sodium Channel Blocking substances, postulated to be PSP toxins (Kodama, 1990, Franca *et al.*, 1996, Levasseur *et al.*, 1996) or their pharmacological cousin TTX (tetrodotoxin) (Noguchi *et al.*, 1986, Simidu *et al.*, 1987, Kodama *et al.*, 1993) and have also recently been implicated in the production of hemolysins (Nayak *et al.*, 1997). The specific toxic action of the culture-filtrate of *A. minutum* remains unresolved at present, however toxicity towards *Artemia* was observed to decline with a reduction in bacterial numbers but this decline was not mirrored by a decline in SCB activity. Although Mascarenhas *et al.* (1995) found that the bacteria associated with *A. minutum* (reported as *A. lusitanicum*) produce toxins dominated by the epimeric pair GTX_1 & GTX_4, as in *A. minutum*, this would not seem to be the toxic principle involved here for three reasons. Firstly, the bacteria were endocellular, at the periphery of the cell, not in the exocellular medium where the toxic principle under investigation here resides. Secondly, pure PSP toxins, in the same concentration and molar ratio (dominated by GTX_1 & GTX_4) as they exist in *A. minutum*, dissolved in seawater, do not affect *Artemia* (see Chapter 3). Thirdly, concentration of SCB toxins did not appear to influence toxicity towards *Artemia*.

Chemical transformation by exocellular bacteria may also play a role in the reduced toxicity of the cell-free *A. minutum* medium towards *Artemia*. We were unable to produce totally axenic cultures, with exocellular bacteria slowly re-establishing themselves in the recovery phases. This has been postulated earlier to be due to the presence of endocellular bacteria that are virtually immune to the effects of exocellular antibiotics. It could be postulated then that the *A. minutum* cells and their associated bacteria are continuing to produce SCBs or an entirely different toxin(s) which are also entering the exocellular medium (refer Chapter 4). Bacteria have been documented as being involved in the transformation of other substances. Beeton & Bull (1989) observed the transformation and detoxification of T-2 toxin (trichothecene-mycotoxin) produced by *Fusarium* and related genera of fungi causing alimentary toxic aleukia and hemorrhagic syndrome) by soil and freshwater bacteria while He *et al.* (1992) reported on bacteria found in soil and lumen fluid as being able to transform deoxynivalenol or vomitoxin. Similarly, Bourne *et al.* (1996) isolated a bacterium, *Sphingomonas* sp., which contained a novel enzymatic pathway which acted on microcystin-LR rendering the compound non-toxic by reducing interaction with the target protein phosphatase. Biotransformation of substances is common for bacteria, and hence the alga itself may be making a toxin that is not a PSP toxin

but this appears to require bacteria to some degree. Either the bacteria are enhancing the production of such a toxin or are using unknown biochemical pathways to transform a substance into an entirely separate toxin that has the ability to kill *Artemia*. Hence, reductions in the amount of bacteria present would give the observed reductions in toxicity of the exocellular, *A. minutum* culture medium.

It is unknown if reducing the bacteria to zero would indeed produce a culture of *A. minutum* whose exocellular medium was non-toxic as the possibility of achieving a state of total axenicity is doubted by many researchers. Also a comprehensive study is required to positively identify what bacteria are present in association with this strain of *A. minutum* and whether pure cultures of these bacteria are toxic.

Chapter 7: General Discussion

7.1 Major Observations

The impetus for the work presented in this thesis was the supected exotoxicity of the PSP dinoflagellate, *Gymnodinium catenatum* towards farmed Atlantic salmon in Tasmanian waters producing the still unresolved clubbing and necrosis gill (CNG) syndrome. However, although exocellular toxicity had been demonstrated for some other gymnodinioids such as *G. breve* and *G. mikimotoi* (Hallegraeff 1991), *G. catenatum* did not appear to share this exotoxic characteristic. The problem of the CNG syndrome was not taken any further past discounting *G. catenatum* as the cause.

At this point the research focus moved to the unexplained exotoxicity of another PSP dinoflagellate, *Alexandrium minutum* towards laboratory reared rotifers and brine shrimp. After careful experimentation using laboratory based exposure experiments the exocellular toxicity of *A. minutum* was confirmed while the toxic compound(s) in the culture-filtrate causing the observed effects was not unambiguously characterised. However, the present work established that xenic cultures of *A. minutum*:

1. produce a potent exotoxin that primarily kills brine shrimp and affects the gills of fish with possible secondary effects appearing in fish blood chemistry, cardiac function and pathology. The toxin is not one of *A. minutum*'s suite of PSP toxins and prolonged exposure results in death of the fish.

2. may produce a substance with NSP-like activity but is not a brevetoxin.

3. excretes a sodium channel blocking substance into its exocellular growth medium. These sodium channel blocking substance(s) are in a concentration in the exocellular medium three orders of magnitude higher than in the endocellular medium.

These points are discussed here in terms of their implications to the overall toxicity of *A. minutum*, and suggestions are made as to the identity of the toxic compound(s) in the *A. minutum* medium based on the available evidence. Other minor points are discussed in the

thesis' main body in the relevant chapters.

7.2 Ichthyotoxicity of *A. minutum*'s Cell-Free Growth Medium

The chemical identity of exocellular toxicity of *A. minutum* towards the experimental animals in this study (*A. salina, R. taparina*) and others described in the literature (Su *et al.* 1993, Bagoien *et al.* 1996, Halim & Labib 1996) remains unresolved. The toxic principle(s) was variable over the growth cycle of the algae, particularly in respect to it's toxicity to *Artemia*, with no specific pattern observed. Although a vast SCB toxin pool was found in the exocellular medium of *A. minutum* it is difficult to ascribe the observed histopathological lesions in the fish and the death of fish and *Artemia* alike to PSP toxins. *G. catenatum*, another PSP toxin producing dinoflagellate was also used to expose *R. taparina*, to both whole cell and culture-filtrate. In all cases with *G. catenatum*, no gill pathology was observed and culture-filtrate of this species was completely non-toxic to *Artemia*. This indicates that although *G. catenatum* produces PSP toxins, these have no effect in the water column on fish tissues and an additional cytotoxin must be being produced by *A. minutum*.

The endocellular PSP toxins and exocellular SCB toxins exhibited a conspicuously similar time course variation. Disregarding any form of PSP toxin transformation into a new toxic species, with a completely changed pharmacology, we are left with the only conclusion that the observed toxic effects of the cell-free *A. minutum* medium are due to another toxin that is not an SCB or one of the known endocellular PSP toxins. In support of this argument, when *Artemia* were challenged with *A. minutum* culture-filtrate over the entire growth cycle of the alga, *Artemia* mortality (tD50) did not appear to be linked to either endocellular PSP toxin concentration or toxin composition, or exocellular SCB toxin concentration. Indeed the peaks in toxicity (tD50) towards *Artemia* were weeks apart from the peaks in both endo/exocellular SCB toxicity, appearing in the mid stationary phase for cultures 1 and 2 but in the early exponential phase in culture 3. Also, heat treatment of the exocellular medium above 80°C caused a reduction in toxicity towards *Artemia*. Heat treatment of PSP toxins normally is concommitant with an *increase* in toxicity. Additionally, no trace of PSP toxins was detected in the exocellular medium of *A. minutum* by absoption onto charcoal columns and subsequent elution and HPLC (A. Negri pers. comm.), indicating a very low concentration (if any) of PSP toxins in the exocellular *A. minutum* medium. Together with the lack of effects of the pure

GTX exposures on both the *Artemia* and the fish, the suggestion of another completely separate toxic principle produced by *A. minutum* appears plausible.

Other toxic species in the genus *Alexandrium* have been observed to produce toxins that are not SCBs. *A. ostenfeldii* and *A. pseudogonyaulax* have been identified as producing the toxic, macrocyclic polyether compounds spirolides and goniodomin, respectively (Wright & Cembella 1998). Gymnodimine, another macrocyclic polyether compound produces similar gill lesions in fish as those reported here from exposure to *A. minutum* culture, as does maitotoxin, another polyether from the ciguatera toxin complex (Terao *et al.* 1996). All of these toxic compounds are related to toxins involved in the NSP syndrome. The histopathological lesions observed in the fish, on exposure to the *A. minutum* culture-filtrate were similar (but not identical) to lesions observed in the gills of red sea bream (*Pagrus major*) (Endo *et al.* 1992) and yellowtail (*Seriola quinqueradiata*) (Toyoshima *et al.* 1985, Doi *et al.* 1981, Shimada *et al.* 1982, Shimada *et al.* 1983) after exposure to the toxic, PbTx-like producing raphidophytes, *C. antiqua*, *C. marina* and the toxic, PbTx producing dinoflagellate *G. breve*. Exposure of fish to these algae produces marked epithelial lifting with variable swelling of the respiratory epithelial lifting, however in the case of *A. minutum* the reverse is the case with the emphasis on respiratory epithelial swelling. Although some authors (Oda *et al.* 1992, Tanaka *et al.* 1994) have indicated that the gill lesions observed in fish exposed to *C. marina* and *C. antiqua* where caused by oxygen radicals, this mechanism cannot be implicated here with *A. minutum*. Oxygen radicals are ephemeral but the exotoxic affect here remained even after culture-filtrate had been stored frozen for several weeks. SCB toxins are not known to cause histopathological lesions in fish gills and that was apparent in the present work with the pure GTX exposures.

In this study we observed signs of cardiac irregularities in many fish exposed to *A. minutum* culture-filtrate. Histopathologically these signs took the form of antemortem blood clots in the heart that appeared ubiquitous in exposed fish but were absent in controls. However, as no electrophysiological experiments were undertaken it cannot be claimed that disruptions to the vagal nerve took place. An extremely high level of potassium, much higher than is normal for this fish, was also detected in the blood plasma. Cardiotoxicity and neuromuscular disturbances can be caused by high levels of blood potassium (Guyton 1991, Greger & Winhorst 1996). It is postulated that the high levels of potassium probably were the result of a combination of

primarily gill damage and subsequent erythrocytic lysis. The rise in potassium from these two factors would then have led to cardiac problems resulting in the eventual death of the fish. The work by Endo *et al.* (1992) on the role of neurotoxins from *C. marina* (PbTx producer) in the induction of cardiac disorder and death in *P. major* indicated that fish are clinically susceptible to toxins in the water column by absorbing them over the gills (Lewis 1992, Munday pers. comm.). However, Endo *et al.* cited vagal nerve depolarization as the aetiology of cardiac dysfunction (the vagus nerve controls heart function, [Carlson 1991]) and death via anoxia from reduced perfusion of the gill by blood. The appearance of both gill damage and cardiac irregularities, similar to intoxication with a polyether toxin offers a tempting analogy. NSP-like activity was suggested by neuroblastoma tissue culture assay based on results gained with this assay using mixtures of pure PSP and NSP toxins (N. Towers, pers. comm.), however PbTx was not detected, although a myriad of other toxins similar to PbTx in both chemistry and pharmacology are known to exist. Wright and Cembella (1998) indicate that only negligible amounts of PbTxs are detected in the culture-filtrate of cells in exponential phase, whereas in stationary phase the proportion of toxin in the culture medium increases as much as 30% of the total (higher presence of disintegrating cells in mature cultures). Mid-stationary phase accounted for the highest toxicity of cell-free *A. minutum* medium to *Artemia* (although this was not the case in culture 3). This high degree of toxicity declined after this point possibly due to a labile nature of the toxic principle(s) but toxicity was also high towards *Artemia* in the mid lag phase of the growth cycle.

Supporting the hypothesis of an unknown, secondary toxic principle in *A. minutum* is the observation that when the bacterial load of *A. minutum* cultures was reduced, similarly was the toxicity of the related cell-free growth medium to *Artemia* decreased. However, the levels of SCB toxins in the same medium, remained the same. Production of SCB toxins (in the form of PSP toxins) by bacteria has been proposed many times in the literature (Kodama *et al* 1993, Levasseur *et al.* 1996, Shimizu *et al.* 1996, Doucette *et al.* 1998). It is entirely possible that in this species of dinoflagellate, bacteria are not involved in PSP toxin production whereas this may be the case with other species, although the problems of gaining a totally axenic culture have already been discussed. The culture-filtrate did not become completely non-toxic as 93% bacterial reduction was the highest level achieved. This again seems to indicate that another toxin is involved as although toxicity to *Artemia* changed, SCB toxin concentration did not. It cannot be concluded with any certainty whether the toxic principle in the exocellular medium is exclusively produced by bacteria, the dinoflagellate or synergistically by both. What appears to

be clear is that the toxic principle is distinct from SCBs (PSP toxins), is not a PbTx (but may have similar properties) and bacteria appear to play a role in its production.

7.3 Dynamics of *A. minutum* SCB Toxicity

With constant advances in technology it is increasingly possible to clarify and resolve phenomena that were difficult to analyse in the past. With the advent of specific toxicological assays, such as the neuroreceptor binding assay developed by G. Doucette *et al.*, it is now possible to assay for exocellular phycotoxins in the water column itself, rather than simply focus on endotoxins via HPLC. The STX neuroreceptor binding assay was used in this study and detected a massive pool of exotoxic SCB toxins in the growth medium of *A. minutum*, hitherto only known to have endotoxic PSP toxins. This is the first time that such a toxic pool has been detected and in such quantity compared to that within the cell. Without any clear clarification as to exactly *what* SCB toxin species are present, i.e. are they indeed PSP toxins, it is difficult to ascribe a role to them. Steidinger & Haddad (1981) considered PSP toxins to be strictly endocellular, with release only occurring under mechanical stress and cellular rupture although, Blanco & Campos (1988) suggest that there may be an increase in cellular permeability during late exponential growth phase accounting for observations of high PSP toxin release during this time. However, the peak in exocellular SCB toxicity was in the lag phase of the culture concurrent with the peak in endocellular PSP toxicity indicating that the excretion of the toxins was most likely an active process. If we assume that they are PSP toxins then we are left with a seeming paradox. When contained within the cell the PSP toxins are potent neurotoxins perhaps having an antiherbivory role as many organisms become affected such as fish (White 1981) and zooplankton (see Chapter 1) after low oral doses but this only seems the case when the cells themselves are consumed (Gosselin *et al.* 1989, White *et al.* 1989, Robineau *et al.* 1991). However, when in the exocellular medium, fish and zooplankton do not appear to similarly be affected. White (1981) indicates that animals do not accumulate PSP toxins from solution. This leaves us with the question of exactly what is the purpose of a large pool of exocellular SCB toxins?

Excretion of cellular compounds is a common feature among aquatic algae (Blanco & Campos 1988). Secreted dinoflagellate compounds are usually soluble organic substances (Fogg 1966, 1983) and this includes many of the known algal toxins. It could be postulated that as SCB

toxins react with membrane bound sodium channels, these being ubiquitous in cells within nature, that it is some form of allelopathy against other algal species however, this did not appear to be the case here as *Tetraselmis suecica* cultures were successfully grown in the presence of *A. minutum* culture-filtrate (Chapter 1) and no such claims of allelopathy are presented in the literature of PSP dinoflagellates as they are for other algal species (eg. *Heterosigma*).

7.4 Future Research

Although much preliminary work has been done to firstly confirm the presence of an unknown, exotoxic principle in cultures of *A. minutum*, and to document its effects, as previously stated the actual chemical identity of the toxic principle remains unknown. This would constitute one of the main priorities of future research. In conjunction with this, the massive pool of SCB toxins detected in the exocellular medium would also constitute a major research thrust of any attempts at clarifying the exotoxicity of this dinoflagellate species.

The identification and quantification of the SCBs in the cell-free culture media of *A. minutum*, would appear to be the logical "next-step" in an ongoing research effort. However HPLC continues to prove unreliable for the detection of substances dissolved in seawater and research efforts are continuing to solve this but, no protocol has yet been satisfactorily developed. In conjunction with this, bioassay guided (fish) fractionation of the cell-free media culture to purify the unknown toxic principles(s) with subsequent mass spectrometry for identification to allow for the final diagnostic analysis using pure toxin exposures to confirm mortality and histopathological effects in original bioassay species.

7.5 Conclusions

Gymnodinium catenatum was conclusively disproved as the cause of the clubbing and necrosis gill syndrome in farmed Atlantic salmon. However, a potent exotoxic principle was discovered in the culture-filtrate of *A. minutum*. Although anecdotal reports of ichthyotoxicity have been reported in the literature concerning *A. minutum*, this is the first definitive proof of such activity. Although the toxic principle's identity remains a mystery we have conclusively proved that it is toxic to brine shrimp and marine fish causing severe gill damage, cardiac

perturbations and eventual death. A pool of SCB substances was also detected in the exocellular medium of *A. minutum* in a vastly greater concentration than inside the cell. This is the first time that such a pool of substances capable of blocking sodium channels has been found in the growth medium of an alga although they do not appear to be PSP toxins as none could be be detected by adsorption onto charcoal columns with subsequent HPLC analysis of charcoal washings (Negri pers comm). Likewise the ichthyotoxic principle(s) in the exocellular medium of *A. minutum* was not thought to be a PSP toxin as pure PSP toxins did not produce any of the pathological lesions observed in flounder nor did they produce mortality in fish or brine shrimp alike. This is the first definitive support of the general assumption that PSP toxins do not cause fish histopathology or death when dissolved in the water column. In support of this an SCA substance with NSP-like activity (but was not a PbTx) was detected in the exocellular medium of *A. minutum* using neuroblastoma assay. No algal species has ever been shown to produce both SCB and SCA substances but the histopathology observed in the flounder was similar to fish exposed to NSP producing algae.

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Appendix 1

G.P. Medium-modified Protocol (GSe)

1. Seawater

Autoclave purified (activated charcoal treatment), filtered seawater in one litre, screw-cap teflon bottles.

2. Stock Solutions

1.	KNO_3		100.0 $gL^{-1}H_2O$
2.	K_2HPO_4		34.8 $gL^{-1}H_2O$
3.	Vitamins	B_{12}	0.1 mg $100ml^{-1}H_2O$
	(fresh soln. every 3 months)	Thiamine HCl	100.0 mg $100ml^{-1}H_2O$
		Biotin	0.2 mg $100ml^{-1}H_2O$
4.	PII Metal Mix	Na_2EDTA	6.0 $gL^{-1}H_2O$
		$FeCl_3.6H_2O$	0.29 $gL^{-1}H_2O$
		H_3BO_3	6.85 $gL^{-1}H_2O$
		$MnCl_2.4H_2O$	0.86 $gL^{-1}H_2O$
		$ZnCl_2$	0.06 $gL^{-1}H_2O$
		$CoCl_2.6H_2O$	0.026 $gL^{-1}H_2O$
		(adjust pH to 7.8-8.0 with NaOH pellets)	
5.	Soil Extract		
6.	Selenium (as selenite)	H_2SeO_3	1.29 mg L^{-1} H_2O

3. To Prepare Nutrient Solution (excluding soil extract)

Make up a working solution of nutrients in distilled water.

For 100ml mix:

10.0ml nitrate stock

5.0ml phosphate stock

5.0ml vitamin stock

25.0ml PII metals

5.0ml selenium stock

50.0ml distilled water

Under aseptic conditions, filter-sterilize nutrient solution, using 0.22µm disposable filter unit, into a sterilized 100ml Schott bottle.

To be used at 2ml per 100ml medium concentration.

4. To Prepare Working Soil Extract Solution

Mix 25ml soil extract and 25ml distilled water.

Autoclave in 100ml Schott bottle

To be used at concentration, 1ml per 100ml medium.

5. Distilled water

Sterilize 400ml distilled water in a 500ml Schott bottle

6. To Prepare Medium

In a sterile 500ml Schott bottle add aseptically:

330ml sterile seawater (1)

70ml sterile distilled water (5)

8ml working nutrient solution (3)

4ml working soil extract solution (4)

Mix:

This medium is now ready to be decanted aseptically into sterile culture flasks.

Modification

34% GSe is prepared in the same way except the sterile distilled water is excluded from the medium i.e. use full strength seawater.

Soil Extract

To Prepare Soil Extract

1. Sift dry soil (not recently treated with fertilizer or herbicide) once through a course sieve, and twice through a fine sieve.
2. Mix 1kg of soil into 2L of distilled water.
3. Autoclave for 60 minutes at 121°C, and cool overnight.
4. Filter through cotton wool packed into glass filter funnel.

5. Centrifuge at 5000rpm. for 20 minutes in 250ml polyethylene centrifuge tubes at 20°C.
6. Dispense the supernatant (50ml aliquots) into 100ml Schott reagent bottles or 100ml media bottles.
7. Autoclave for 15 minutes at 121°C.
8. After cooling, wrap caps with parafilm.
9. Store sterile soil extract at 4°C (cold room or refrigerator).

Appendix 2

Brine Shrimp Hatching and Growth

Artemia eggs (Lot. No. 95385, "*Artemia* Revolution", NT Laboratories, Kent, England, TN12, 5HF) were obtained from a local aquarium shop and kept in a light proof container at 5°C, to maintain hatching viability and to suppress any spontaneous hatching. *Artemia* require a stable temperature of approximately 23.5°C for hatching. Hence, approximately 0.5ml (three drops) of egg solution was placed in a hatchery made from an upturned polyethylene bottle with the bottom removed and the neck fitted with a bung containing an airstone (Fig. 1). The hatchery was filled with approximately 600ml of clean seawater (approximately 30 ppt. salinity) and then placed in a water bath consisting of a 12L fish aquarium, half filled with water, and heated to the required temperature of 23.5°C by a standard aquarium-thermostatic heater (Super polyethylene, polyethylenes International, PTY, LTD, N11417).

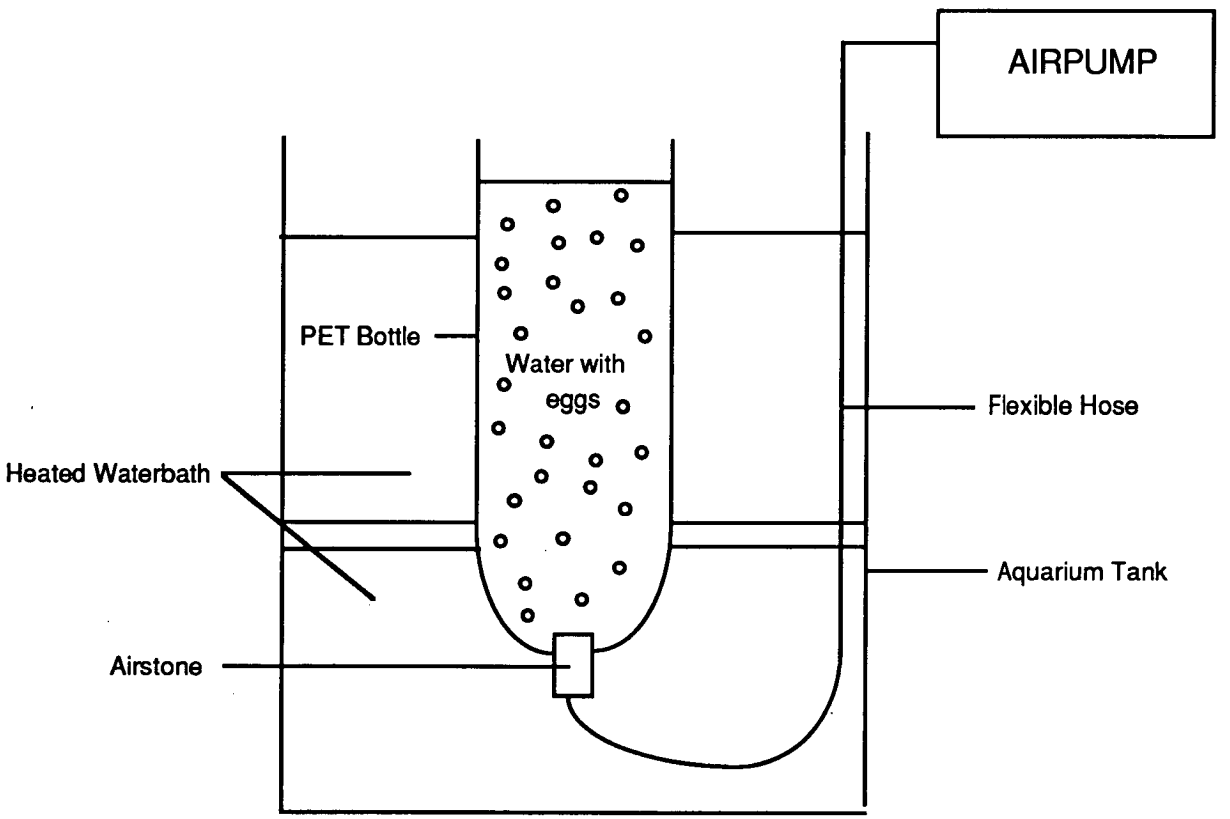

Figure 1: Hatchery constructed for the growth of *A. salina* to 4-day old metanauplii stage for use in bioassays.

The *Artemia* used were decapsulated which gives a higher hatch yield however, the eggs lose their natural buoyancy and so need to be kept in suspension in order for them to hatch successfully. To accomplish this, aeration was from the bottom of the hatchery, with a standard aquarium aerator (Dyna-one). *Artemia* eggs hatched in approximately 48 hours into first day nauplii (approx. 0 5mm in length). All work was conducted with the 4-day old meta-nauplii (approx 1.5mm in length) and during this time it was not necessary to feed *Artemia.*

For removal, the hatchery was illuminated from the side by a 60 watt tungsten lamp. This caused a phototropic response in the *Artemia,* with large numbers aggregating as close to the light source as possible. They were then simply transferred to experimental conditions via a long tipped, pasteur pipette. *Artemia* could be easily counted as they were moved into the exposure plates and there appeared to be no significant handling injury observed.

Artemia Bioassays

Sterile micropates (24 wells with lid, IKAWI), each well having an approximate 2.5ml volume, were used as exposure microaquaria. Wells were filled with 2.0ml of exposure fluid (whole cell culture, cell-free culture, seawater etc.), the number of wells filled depending on the duration of the exposure (one well per reading). To each aquaria was then added a known number of *Artemia* (usually 5-10) from the hatchery using a pipette. Exposure wells were left at room temperature in the dark (covered with black cardboard). Depending on the time sequence decided, each well was read sequentially. Usually readings were taken either on the hour or every second hour. Hence the first well was read at the first reading, the second well on the second reading and so on. At each reading the "current" well was observed for *Artemia* death. Death was concluded if the *Artemia* did not respond to a gently prodding glass pipette. Each round of exposures contained a seawater (plus nutrients) control. tD50's were determined using PROBIT analysis (Systat).

Appendix 3

Tissue Staining

Heamatoxylin/Eosin

1.	Xylene	3 min
2.	Xylene	1 min.
3.	100% Ethanol	3 min
4.	70% Ethanol	3 min
5.	50% Ethanol	3 min
6.	Water	2 min
7.	Heamatoxylin	2 min
8.	Running tap water	>5 min
9.	50% Ethanol	3 min
10.	70% Ethanol	3 min
11.	100% Ethanol	3 min
12.	Eosin	4 min
13.	100% Ethanol	1 min.
14	100% Ethanol	1 min.
15.	Xylene	3 min
16.	Xylene	indefinite (mount)

Heamatoxylin: Mayers (Histo Labs 0494124)
Eosin: 1% aqueous (Histo Labs 1293105)
DePex Mounting Medium: Gurr 36125.

Alcian Blue

1.	Xylene	3 min
2.	Xylene	1 min.
3.	100% Ethanol	3 min
4.	70% Ethanol	3 min

5.	50% Ethanol	3 min
6.	Water	2 min
7.	Alcian Blue 2.5	30 min
8.	Rinse 3% Acetic Acid	rinse
9.	Running Tap Water	3 min.
10.	50% Ethanol	3 min
11.	70% Ethanol	3 min
12.	100% Ethanol	3 min
13.	Eosin	4 min
14.	100% Ethanol	1 min.
15	100% Ethanol	1 min.
16.	Xylene	3 min
17.	Xylene	indefinite (mount)

Alcian Blue pH 2.5

Alcian Blue (Sigma A-2899)	1.0g
3% aqueous acetic acid	100ml

keeps for several years.

DePex Mounting Medium: Gurr 36125.

Appendix 4

PSP Toxin Analysis by HPLC (Oshima *et al.* 1988)

The detection of PSP toxins by HPLC is based on the method utilizing reverse phase separation by ion-pair chromatography, followed by post-column oxidation of the underivatised toxins in alkaline solution, then finally acidification and fluorescence detection (Oshima *et al.* 1988).

Apparatus & Reagents

The HPLC system used in this study consisted of a Waters WISP 710B LC autosampler, a Waters 6000A solvent delivery system (Mobile Phase), ICI LC1110 HPLC pump (oxidising reagent at 0.4 ml $min.^{-1}$), Waters M-45 HPLC pump (Acidifying Reagent at 0.4 ml $min.^{-1}$) and a teflon reaction coil (0.5mm id x 10m) at 65°C. The eluted PSP toxins were detected and quantified using an Hitachi F-1000 fluorescence spectrophotometer (Ex 330nm & Em 390nm) and a CHROMATOPAC C-R3A, Shimadzu integrator.
Three different isocratic runs using specified mobile phases were used in the separation of the three groups of PSP toxins.

C toxins: 1.5 mM tetrabutyl ammonium phosphate in milli Q water adjusted to 6.8 with 0.05N acetic acid (0.8ml $min.^{-1}$). Develosil C8-5 column, 4.6 x 250mm, Nomura Chemical Co.

Gonyautoxins: 3 mM octanesulfonic acid and 9mM phosphoric acid in milli Q water, pH adjusted to 7.0 with 1N NH_4OH (0.8ml $min.^{-1}$). Nova Pak C8 column (Waters), 3.9 x 150mm, 60A.

Saxitoxins: 4 mM Heptanesulphonic acid and 20mM phosphoric acid in milli Q water, pH adjusted to 7.0 with 1N NH_4OH. Plus 20ml of acetonitrile (CH_3CN) (0.8ml $min.^{-1}$). Nova Pak C8 column (Waters), 3.9 x 150mm, 60A.

The oxidising reagent consisted of 7 mM periodic acid and 50mM sodium hydrogen ortho-phosphate in milli Q water, pH adjusted to 9.0 with 1N NaOH. It was prepared fresh daily and was added to the LC stream after column separation at a flow rate of 0.4ml $min.^{-1}$. The acidifying reagent, added to the LC stream after the reaction coil, was 0.5M acetic acid at a flow rate of 0.4ml $min.^{-1}$.

Appendix 5

Seawater agar with vitamins (SWAV) - general medium for marine bacteria

Yeast extract 1.0g	
Bacteriological peptone	1.0g
Bacteriological agar	15.0g
Vitamin solution	2.5ml
Filtered seawater (10μm)	1000ml

Sterilize by autoclaving at 121°C for 15 min. Pour agar into petri dishes and allow to solidify.

Vitamin solution

Thiamine HCl	20.0mg $100ml^{-1}$ distilled water
Biotin	0.1mg $100ml^{-1}$ distilled water
Vitamin B12	0.1mg $100ml^{-1}$ distilled water

Store at 4°C. Replace every 3 months.

Appendix 6

Glossary of Terms:

allelopathic: the effect of one living plant on another.

anaemia: shortage of haemoglobin, the oxygen carrying pigment in erythrocytes.

antemortem: occurring before death.

bradycardia: a reduction in heart rate from the normal level.

cardiac arrythmia: a disturbance in the natural beating rhythm of the heart.

cytoplasmic shrinkage: shrinkage of the cytoplasm away from the cell membrane.

cytotoxic: toxic to cells.

ectoparasites: parasites on the external surface of an organism.

edema: swelling caused by an abnormal accumulation of fluid in interstitial tissues.

endotoxin: toxin contained within cellular cytoplasm.

epithelium: any cellular tissue covering a free surface or lining a tube or cavity.

erythrocytes: red blood cells.

exotoxin: soluble, excreted toxin.

exudate: substance given out by exudation.

fatty degeneration: excessive accumulation of fat (mostly neutral fat) in the cytoplasm, and is often accompanied by nuclear atrophy. Usual paraffin sections exhibit foamy structures in the cytoplasm of the liver cells where fat was originally present.

gill cavity: cavity or space behind the operculum where the gills sit.

hemagglutinative: a substance causing the rapid agglutination of blood fluid.

hemolytic: breakdown of erythrocytes (red blood cells).

haemorrhage: the escape of blood from a blood vessel.

hyperkalaemia: raised plasma potassium levels.

hyperplasia: overgrowth, excessive or hyperplastic development due to increase in the number of cells.

hypertrophy: excessive growth due to increase in the size of cells.

interlaminar cells: cells between the gill secondary lamellae, on the primary lamellae.

ichthyotoxic: toxic to fish or other marine fauna.

ion channel: a specialized protein molecule that permits specific ions to enter or leave cells *Voltage gated* ion channels open or close according to the value of their membrane potential.

lesion: an injury of tissue or organ resulting in impairment of function.

leucocyte: a colourless often amoeboid cell of blood, white blood cell, plasmacyte, amoebocyte.

lumen: the cavity of a tubular part or organ, central cavity of a plant cell.

lipophilic: fat soluble.

macrophage: a large phagocytic cell, fixed or wandering, a large mononuclear leucocyte, a histiocyte, clasmatocyte, pericyte, endotheliocyte.

microsporidian: intracellular parasite with ovoid spores less than 10 microns, parasitic disease, not usually fatal, common in wild fish.

mucocyte: cell which produces mucous.

mucosa: the innermost layer of the gut wall consisting of 3 layers, namely the inner epithelium with digestive glands, the lamina propria, and the muscularis mucosa; the epithelium and lamina propria are together called the mucous membrane (mucous coat) or the whole mucosa can be called the mucous membrane.

neurotoxic: toxin affecting neural tissue.

necrosis: the death of cells or tissues.

operculum: a lid or covering flap, gill cover of fishes.

P-wave: the electrical activity (portion of an electrocardiogram) associated with depolarization of the atrium.

pantothenic acid: an acid of the vitamin B complex, sometimes called vitamin B_3 or B_5, that is found in all living tissues, often combined as in coenzyme A, and is necessary for growth in various animals.

pathogen: any disease causing microorganism.

pharynx: a musclo-membranous tube extending from the undersurface of the skull to the level of the 6th cervical vertebrae; gullet or anterior part of alimentary canal following buccal cavity.

pillar cell: hourglass cell, cells separating the gill-blood capillaries.

post mortem autolysis: breakdown of cells after death.

pyknosis: a thickening, especially degeneration of a cell in which the nucleus shrinks in size and the chromatin condenses to a solid, structureless mass or masses.

QRS complex: the electrical activity (portion of an electrocardiogram) associated with depolarization of the ventricle.

respiratory epithelial distance: distance from the inside surface of the gill-blood capillary to the outer surface or tissue/water interface of the gill, the distance O_2 must pass through to enter the blood stream.

sodium channel: transmembrane channel allowing passage of sodium ions.

telangiectasis: ballooning of blood vessels similar to aneurysms.

teleosts: a group of advanced actinopterygians including the majority of modern fishes, existing from the Jurassic to the present day, having scales of thin bony plates covered by epidermis, a homocercal tail, a hydrostatic air bladder, and no spiracle or spiral valve in the gut.

vacuolation: the formation of vacuoles, appearance or formation of drops of clear fluid in growing or ageing cells